

Remarks

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 23, 26-33, 36-43, 46-53, 58-65, 70-76 and 93-110 are pending in the application, with 23, 33, 43, 53, 65, 93, 99 and 105 being the independent claims. Claims 24, 25, 34, 35, 44, 45, 54-57, 66-69 and 77-92 are sought to be cancelled without prejudice to or disclaimer of the subject matter therein. New claims 93 to 110 are sought to be added. These claims are directed to the subject matter of Group II, *i.e.*, polynucleotide sequence encoding the EDG-1-like G-protein coupled receptor, and support for the new claims can be found in the claims as originally filed and throughout the specification. Accordingly, new claims 93 to 110 do not require a new search or substantial examination.

These changes are believed to introduce no new matter, and their entry is respectfully requested. In addition, it is believed that these amendments will put the case in condition for allowance or better form for consideration on appeal.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Objection to the Specification

The Examiner objected to the specification due to an informality. (*See* Office Action, page 2, ¶ 3.) In particular, the Examiner indicated that "[t]he specification must be amended to 'Brief Description of the Drawings' as required by MPEP 608.01(f) to over come the objection." (Office Action, page 2, ¶ 3.). Applicants have amended the

specification in accordance with the Examiner's requirements and MPEP 608.01(f). Hence, this objection is overcome. Applicants respectfully request that the Examiner withdraw the objection.

Rejections under 35 U.S.C. § 112, Second Paragraph

The Examiner rejected claims 57, 69, 77-81 and 83 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite. (*See* Office Action, page 2, ¶ 4.) Specifically, the Examiner asserted that claim 77 "remains indefinite because it is not clear which amino acids comprise the transmembrane region domain of SEQ ID NO: 4 so as to allow the metes and bounds of the claims [sic] to be determined." (Office Action, page 2, ¶ 4.)

Solely to advance prosecution, and not in acquiescence to the Examiner's rejection, Applicants have canceled claim 77, thereby rendering this rejection moot. Applicants respectfully request that the Examiner withdraw the rejection.

Claims 57 and 69 were rejected as allegedly being indefinite "because it is unclear when an antibody has 'specificity' for the polypeptide of SEQ ID NO:4 so as to allow the metes and bounds of [the] claim to be determined." (Office Action, page 4, ¶ 4.) Solely to advance prosecution, and not in acquiescence to the Examiner's rejection, Applicants have canceled claims 57 and 69, thereby rendering this rejection moot. Applicants respectfully request that the Examiner withdraw the rejection.

The Examiner rejected claims 78-81 and 83 as being indefinite for depending on allegedly indefinite claims. (*See* Office Action, page 4, ¶ 4.) Solely to advance prosecution, and not in acquiescence to the Examiner's rejection, Applicants have

canceled claims 78-81 and 83, thereby rendering this rejection moot. Accordingly, Applicants respectfully request that the Examiner withdraw the rejection.

Rejections under 35 U.S.C. § 101

The Examiner rejected claims 23-29, 31, 33-39, 41, 43-49, 51, 53-61, 63, 65-73, 75, 77-81 and 83 under 35 U.S.C. § 101 "because the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility." (Office Action, page 4, ¶ 5.) Contrary to the Examiner's position, the claimed invention is supported by a specific, substantial and credible asserted utility.

The specification asserts specific and substantial utilities for the presently claimed invention, e.g. the diagnosis and treatment of cancer (specification at 8), screening for antagonists and/or agonists (*id.* at 21) which may be used for the treatment of cancer (*id.* at 22-24), and the diagnosis of the mutations associated with cancer or susceptibility to cancer (*id.* at 27).

Regarding the specificity of an asserted use, Applicants note that the Utility Guidelines define "specific utility" as a utility that

is *specific* to the subject matter claimed. This contrasts with a *general* utility that would be applicable to the broad class of the invention. . . . For example, indicating that a compound maybe useful in treating unspecified disorders, or that the compound has "useful biological" properties, would not be sufficient to define a specific utility for the compound.

MPEP § 2107.01 (I.) at 2100-32 (May 2004).

Applicants assert that the specification does not assert that the claimed invention "maybe useful in treating unspecified disorders." Rather, in view of the expression sites

of the claimed polynucleotides, its sequence as well as the knowledge of other G-protein coupled receptors, among other things, it was asserted in the specification that the claimed invention possesses utility in a specified disease state, *i.e.*, human cancer. Further, the use of these EDG-1-like molecules to treat, for example, cancer is a specific use that is not generally applicable to all G-protein coupled receptors, much less to all proteins. Accordingly, it follows that the statement of utility is clearly specific under the Utility Guidelines.

Applicants also respectfully re-emphasize that the specification discloses at least one specific and substantial utility for the EDG-1-like G-protein coupled receptor. A substantial utility is one that defines a "real world" use. In addition, "any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a 'substantial' utility." MPEP § 2107.01 at 2100-33 (May 2004). The use of EDG-1-like G-protein coupled receptor molecules to treat and/or diagnose, for example, cancer, is a substantial utility as it provides a benefit to the public. Thus, at least one asserted use for the EDG-1-like G-protein coupled receptor is specific and substantial, as well as credible, as discussed further below.

The Examiner further asserted that:

Applicants' reference to *post filing art cannot be used to establish utility for the claimed invention*. . . At the time of filing [the] instant Application, the specification nor prior art supported the assertion that antagonists of the EDG-1-like G protein coupled receptor could be used to treat cancer or the polynucleotides of the invention could be used for the detection of cancer. The functionality of claimed EDG-1-like G protein coupled receptor was unknown.

(Office Action, page 6, ¶ 1.) (*emphasis added*)

Applicants assert that they are not using post filing date art to establish utility for the claimed invention. Rather, Applicants have brought post filing date art to the attention of the Examiner to show that one of the substantial utilities specifically asserted in the specification is in fact credible. Applicants note that both the MPEP and Federal Circuit indicate that post-filing art may be used to *substantiate* a specific and substantial utility asserted in the specification. If a specific and substantial utility is asserted in the specification, a post-filing date reference setting forth test results substantiating the utility "pertains to the accuracy of a statement already in the specification. . . . It does not render an insufficient disclosure enabling, but instead goes to prove that the disclosure was in fact enabling when filed (i.e., demonstrated utility)." *In re Brana*, 51 F.3d 1560, 1567 n.19 (Fed. Cir. 1995).

In addition, the MPEP § 2107.02 states that "[i]n appropriate situations the Office may require an applicant to substantiate an asserted utility for a claimed invention. See *In re Pottier*, 376 F.2d 328, 330, 153 USPQ 407, 408 (CCPA 1976)." Furthermore:

If the applicant responds to the *prima facie* [35 U.S.C. § 101] rejection, Office personnel should review the original disclosure, any evidence relied upon in establishing the *prima facie* showing, any claim amendments, and *any new reasoning or evidence provided by the applicant in support of an asserted specific and substantial credible utility*.

MPEP § 2107.02 VI (May 2004) (*emphasis added*). Thus, Applicants may provide post filing art or "any new reasoning or evidence," to substantiate an asserted utility. Applicants respectfully assert, therefore, that the remarks and arguments regarding utility

made in the Amendment and Reply Under 37 C.F.R. § 1.111 filed January 10, 2002, are fully applicable and are incorporated by reference herein. Applicants also provide new reasoning and evidence.

This asserted specific and substantial utility of the EDG-1-like G-protein coupled receptor is supported by its significant similarity to the G-protein coupled receptor EDG-6, also known as S1P₄ receptor. *See* Van Brocklyn *et al.*, *Blood* 95: 2624-2629 (2000). The EDG-6 receptor is nearly identical to the EDG-1-like G-protein coupled receptor, differing in only 4 amino acids out of 384. Expression of EDG-1-like G-protein coupled receptor mRNA is found in lymphocytes and lymphoid, hematopoietic and lymphoid-associated tissue such as the lung. (*See* specification at 7.) EDG-6 is likewise expressed in lymphocytes, lymphoid tissue, hematopoietic tissue and lung.

The art confirms that EDG-6 is associated with cancer. EDG-6 is a receptor for Sphingosine-1-Phosphate (SPP or S1P). *See* Van Brocklyn *et al.* Invasion of T-lymphoma cells into a fibroblast monolayer is dependent on SPP receptor-mediated RhoA and PLC signaling pathways that lead to pseudopod formation and enhance infiltration. *See id.* at 2628. It is well known in the art that chemotaxis and invasion are important events in the spreading of cancer.

Further, EDG-6 is a receptor for SPP and mediates this SPP-dependant cancer-associated chemotactic response. *See* Gräler *et al.*, *J. Cell. Biochem.* 89:507-519 (2003) (Exhibit A). "CHO-K1 cells ectopically expressing [EDG-6] potently activate the small GTPase Rho and undergo cytoskeletal rearrangements, including stress fiber formation and cell rounding, upon [SPP] stimulation. Overexpression of [EDG-6] in Jurkat T cells induces pertussis toxin-sensitive cell motility even in the absence of exogenously added

[SPP]." *Id.* at 507. The importance of EDG-6 in SPP-induced cell migration has been confirmed by a second group, who also reported that motility was mediated through the Rho GTP-ase Cdc42. *See* Kohno *et al.*, *Genes to Cells* 8: 685-697 (2003) (Exhibit B).

As a result, one of ordinary skill in the art would consider that the presently claimed EDG-1-like G-protein coupled receptor is important in the motility, invasion and chemotaxis of lymphocytes and that these functions are relevant to the spread of cancer. Accordingly, the post filing art submitted herewith provides evidence as to the accuracy, *i.e.*, credibility, of at least one specific and substantial utility as asserted in the specification.

Regarding the credibility of an asserted utility, the Utility Guidelines provide as follows:

Where an applicant has specifically asserted that an invention has particular utility, that assertion cannot simply be dismissed by Office personnel as being "wrong," even when there may be reason to believe that the assertion is not entirely accurate. Rather, Office personnel must determine if the assertion of utility is credible (*i.e.*, whether the assertion of utility is believable to a person of ordinary skill in the art based on the totality of evidence and reasoning provided).

MPEP § 2107.02 (III.)(B.) at 2100-40 (May 2004). In other words, the Examiner "must provide evidence sufficient to show that the statement of asserted utility would be considered 'false' by a person of ordinary skill in the art." MPEP § 2107.02 (III.)(A.) at 2100-40 (May 2004). Applicants respectfully submit that the Examiner has not met this burden.

Applicants have asserted that the claimed polynucleotides can be used, for example, in a diagnostic assay for detecting diseases or susceptibility to diseases, such as

tumors and cancers, related to the presence of mutated G-protein coupled receptor genes (specification at 27), and to produce polypeptides which can be used to raise monoclonal antibodies for the diagnosis and/or treatment of cancer (specification at 8 and 22-23).

Applicants submit that the above assertions are not only specific and substantial, but credible as well, *i.e.*, the assertion is *at least believable* to, and would not be considered *false* by, a person of ordinary skill in the art. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw the rejection of the claims under 35 U.S.C. § 101.

Rejections under 35 U.S.C. § 112, First Paragraph

The Examiner rejected claims 23-29, 31, 33-39, 41, 43-49, 51, 53-61, 63, 65-73, 75, 77-81 and 83 under 35 U.S.C. § 112, first paragraph "since the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility for the reasons set forth above, one skilled in the art would clearly not know how to use the claimed invention." (Office Action, pages 9-10, ¶ 6.) This rejection under 35 U.S.C. § 112 therefore depends on the prior rejection under 35 U.S.C. § 101. As the rejection under 35 U.S.C. § 101 is overcome for reasons outlined above, this rejection under 35 U.S.C. § 112 is similarly overcome. Consequently, Applicants respectfully request that the Examiner reconsider and withdraw the rejection.

The Examiner also rejected claims 23-29, 31, 33-39, 41, 43-49, 51, 53-61, 63, 65-73, 75, 77-81 and 83 under 35 U.S.C. § 112, first paragraph, "as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application

was filed, has possession of the claimed invention." (Office Action, page 10, ¶ 7.) Specifically, the Examiner asserted that Applicants' previous arguments that "polynucleotides at least 90% identical to the specific polynucleotide will show activity" are "not persuasive," as "[t]here is no description of the conserved regions, which are critical to the structure, and function of the genus claimed." (Office Action, page 11, ¶ 7.) As a result, the Examiner asserted that "[t]he claims encompass billions and billions polynucleotides encoding proteins which are structurally and functionally unrelated to the protein of SEQ ID NO:4." (*Id.*) The Examiner further asserted that only the use of SEQ ID NO: 3 or SEQ ID NO: 4 meet the written description requirements of 35 U.S.C. § 112. (*See id.*)

As set forth in the Amendment and Reply of April 9, 2002, Applicants respectfully disagree with the Examiner. However, solely to advance prosecution, and not in acquiescence to the Examiner's rejection, Applicants have canceled claims 24, 25, 34, 35, 44, 45, 54-57, 66-69 and 72-79 and have amended independent claims 23, 33, 43, 53 and 65 to remove language directed to percent identity, thereby rendering the rejection moot. Thus, Applicants respectfully request that the Examiner reconsider and withdraw this rejection.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the
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Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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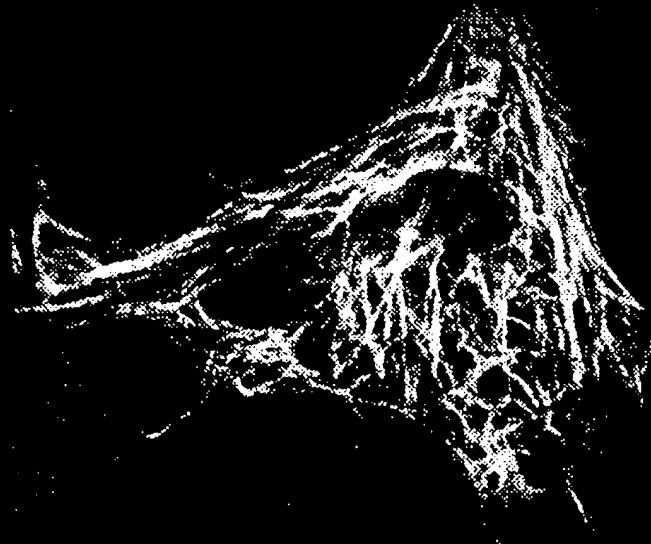
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The Sphingosine 1-Phosphate Receptor S1P₄ Regulates Cell Shape and Motility via Coupling to G_i and G_{12/13}

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Abstract Sphingosine 1-phosphate (S1P) receptors represent a novel subfamily of G-protein-coupled receptors binding S1P specifically and with high affinity. Although their *in vivo* functions remain largely unknown, *in vitro* extracellular application of S1P induces distinct S1P receptor-dependent cellular responses including proliferation, differentiation, and migration. We have analyzed signaling pathways engaged by S1P₄, which is highly expressed in the lymphoid system. Here we show that S1P₄ couples directly to G_α_i and even more effectively to G_α_{12/13}-subunits of trimeric G-proteins, but not to G_α_q unlike other S1P receptors. Consequently, CHO-K1 cells ectopically expressing S1P₄ potently activate the small GTPase Rho and undergo cytoskeletal rearrangements, inducing peripheral stress fiber formation and cell-rounding, upon S1P-stimulation. Overexpression of S1P₄ in Jurkat-T-cells induces pertussis toxin-sensitive cell motility even in the absence of exogenously added S1P. In addition, S1P₄ is internalized upon binding of S1P. The capacity of S1P₄ to mediate cellular responses, such as motility and shape change through G_α_i- and G_α_{12/13}-coupled signaling pathways may be important for its *in vivo* function which is currently under investigation. *J. Cell. Biochem.* 89: 507–519, 2003. © 2003 Wiley-Liss, Inc.

Key words: sphingosine 1-phosphate; endothelial differentiation gene; G protein-coupled receptor; lymphocyte; immune system

Sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) are known as cell mitogens that are capable of inducing diverse cellular responses like proliferation and differentiation, chemotaxis, cell rounding, and tumor invasion (reviewed in Chun et al., 1999; Moolenaar,

1999). Recently, eight G protein-coupled receptors could be identified that bind specifically S1P and LPA as ligands [Hecht et al., 1996; An et al., 1997, 1998a; Lee et al., 1998; Bandoh et al., 1999; Im et al., 2000; Van Brocklyn et al., 2000]. Five of them (S1P_{1–5}) bind S1P [An et al., 1997; Lee et al., 1998; Im et al., 2000; Van Brocklyn et al., 2000], whereas three of them (LPA_{1–3}) are receptors for LPA [Hecht et al., 1996; An et al., 1998a; Bandoh et al., 1999]. Since most cells express more than one receptor for S1P or LPA, heterologous overexpression systems and antisense-RNA studies were used to distinguish between signaling events and cellular responses of each of these receptors [Goetzl et al., 1999; Kon et al., 1999].

The S1P receptors analyzed so far are able to activate the mitogen-activated protein kinases (MAP kinases) ERK1/2 and trigger the

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release of calcium from intracellular stores by activating phospholipase C (PLC) [Okamoto et al., 1998, 1999; An et al., 1999; Gonda et al., 1999; Sato et al., 1999; Im et al., 2000; Van Brocklyn et al., 2000; Yamazaki et al., 2000]. Upon stimulation, S1P₁ and S1P₅ exert a negative effect on the level of cyclic AMP (cAMP) [Zondag et al., 1998; Im et al., 2000], whereas binding of S1P to S1P₂ and S1P₃ raise the cAMP level [Kon et al., 1999]. In addition, it has been shown that S1P₁ undergoes agonist-induced receptor internalization that is thought to be part of the desensitization/resensitization cycle after binding of the specific ligand S1P [Lee et al., 1998; Liu et al., 1999].

With regard to phenotypical responses it has been demonstrated that S1P₁ and S1P₃ induce cell migration towards an S1P stimulus whereas S1P₂ inhibits cell migration and membrane ruffling [Kon et al., 1999; Okamoto et al., 2000]. S1P₁ is involved in angiogenesis as well as in other developmental processes like formation of the embryonic skeletal system and neuronal differentiation [Hla and Maciag, 1990; Liu and Hla, 1997; Liu et al., 2000]. The expression of S1P₂ is enhanced in rat brain during embryogenesis, suggesting a role in early differentiation of neuronal cells and axon development [MacLennan et al., 1997].

Recently we isolated S1P₄ from in vitro differentiated dendritic cells [Gräler et al., 1998]. Because of its predominant expression in cells and tissues of the lymphoid system we suggest that S1P₄ may play an important role in immune surveillance [Gräler et al., 1999]. Here we show the direct coupling of S1P₄ to G_ai and G_a_{12/13}-subunits of trimeric G-proteins. We provide evidence for pertussis toxin (Ptx)-sensitive PLC activation and cytoskeleton rearrangements as corresponding downstream signaling events. It turned out that S1P₄ is a potent activator of G_a_{12/13} and of the small GTPase Rho, respectively. Stimulation of S1P₄ expressing cells induces enhanced peripheral stress fiber formation and cell rounding. In addition, an increased Ptx-sensitive cell motility of stably transfected unstimulated Jurkat cells as well as an agonist-induced internalization of S1P₄ was observed.

MATERIALS AND METHODS

Materials

DMEM, RPMI, and FCS were purchased from Biochrom (Terre Haute, IN), cell culture

supplements, Ptx, and G418 were from Gibco/BRL (Rockville, MD). PBS was from Seromed (Berlin, Germany), other chemicals were purchased from Merck (Poole, Dorset, UK). myo-[³H]inositol (18.6 Ci/mmol) was from Amersham Pharmacia Biotech (Piscataway, NJ), [^{2,8}-³H]adenine and [α -³²P]GTP (3,000 Ci/mmol) were purchased from NEN Life Sciences (Boston, MA). Wildtype- and N19-RhoA constructs in pcDNA3.1 were a kind gift from Dr. Yi Zheng, University of Tennessee, TN. S1P and LPA were purchased from Sigma-Aldrich (St. Louis, MO). Other sources are mentioned in the text.

Cell Culture and Transfection

Chinese hamster ovary cells (CHO-K1, ATCC CCL-61) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FCS). Human embryonic kidney cells (HEK293, ATCC CRL-1573) were cultured in DMEM containing 10% FCS. The human leukemia T-cell line Jurkat (ATCC TIB-152) was cultured in RPMI medium containing 2 mM L-glutamine and 10% FCS. The S1P₄ expression plasmid RC/CMV (Invitrogen, Carlsbad, CA) containing the C-terminal myc-epitope-tagged human or murine S1P₄ or wildtype-RhoA and N19-RhoA constructs in pcDNA3.1 (Invitrogen) were transfected into CHO-K1 or Jurkat cells by electroporation (20 µg DNA; 5 × 10⁶ cells in 800 µl PBS-d; CHO-K1: 250 V, 1,070 µF; Jurkat: 280 V, 1,070 µF) and into HEK293 cells by calciumphosphate precipitation as described previously [Emrich et al., 1993]. Transfection efficiencies were typically 20–35%, checked by fluorescence-activated cell sorting (FACS) analysis.

Fluorescence-Activated Cell Sorting (FACS) Analysis

Construction, expression, and FACS-analysis of the myc-epitope (spacer-epitope: PGGSGP-EQKLISEEDLL) with the murine anti-myc-epitope antibody 9E10 was performed as described previously [Emrich et al., 1993]. The murine S1P₄ receptor construct was also detected with the newly generated monoclonal rat antibodies 2A1, 6D7, and 8B4, directed against the N-terminal part of the murine S1P₄ receptor. Goat-anti-rat-R-phycoerythrin (PE) and donkey-anti-mouse-PE antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) were used for the detection of unlabeled antibodies from rat and mouse.

Photolabeling of Receptor-Activated G-Proteins

Photolabeling of membrane G-proteins derived from CHO cells and immunoprecipitation were performed as described previously [Grosse et al., 2000]. In brief, [α -³²P]GTP azidoanilide was synthesized and purified as described [Offermanns et al., 1991]. CHO-K1 membranes (200 μ g of protein per assay tube) were incubated at 30°C in a buffer containing 0.1 mM EDTA, 10 mM MgCl₂, 30 mM NaCl, 1 mM benzamidine, 50 mM HEPES (pH 7.4), and for G α_i coupling also with 10 μ M GDP. Samples were incubated for 3 min (G α_i) or 30 min (G $\alpha_{q/11}$, G $\alpha_{12/13}$, G $\alpha_{15/16}$) with 10 nM [α -³²P]GTP azidoanilide (130 kBq per tube) in the absence or presence of 1 μ M S1P. The final assay volume was 120 μ l. After stopping the reaction by cooling the samples on ice, samples were centrifuged at 4°C for 5 min at 12,000g and pellets were resuspended in 60 μ l of the buffer described above supplemented with 2 mM glutathione. Suspended membranes were irradiated for 10 s at 4°C with a 254 nm UV-lamp (Vilber Lourmat, Torcy, France) [Offermanns et al., 1991]. For immunoprecipitation, photolabeled membranes were pelleted and solubilized in 40 μ l of 2% SDS at room temperature. Thereafter, 120 μ l of precipitating buffer (1% Nonidet P-40, 1% desoxycholate, 0.5% SDS, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 10 mM Tris/HCl, pH 7.4) was added. Solubilized membranes were centrifuged at 4°C for 10 min at 12,000g to remove insoluble material, and 50 μ l of 10% protein A-Sepharose beads (Sigma-Aldrich) was added for preclearing of the lysates. After 1 h, Sepharose beads were removed by centrifugation, and 30 μ l of the following antisera were added to the supernatants: Z811 (G $\alpha_{q/11}$), AS233, and AS343 (G $\alpha_{12/13}$), AS266 (G α_i , common) [Laugwitz et al., 1994], and anti-G $\alpha_{15/16}$. After overnight incubation of samples at 4°C at constant rotation, 60 μ l of 10% protein A-Sepharose beads were added, and samples were incubated for additional 3 h. After washing of Sepharose beads, immunoprecipitated G-protein α -subunits were separated on 13% polyacrylamide gels and visualized by autoradiography of dried gels with Kodak X-Omat AR-5 films (Eastman Kodak, Rochester, NY) or with a phosphorimaging screen (Fuji, Stamford, CT).

Measurement of Intracellular Inositol Phosphate and cAMP Accumulation

For cAMP measurements, CHO-K1 cells were seeded into six-well plates (8×10^5 cells/well) 2 days prior to functional assays. [2,8-³H]adenine (2 μ Ci/ml) was added to the growth medium. After a labeling period of 24 h, cells were washed once in PBS, followed by a 20 min preincubation with serum-free DMEM containing 1 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich). Subsequently, cells were stimulated with 1 μ M S1P or 50 μ M forskolin for 1 h. Reactions were terminated by addition of 2 ml 5% trichloroacetic acid. For IP determinations, CHO-K1 cells were seeded into 12-well plates (3×10^5 cells/well) 3 days prior to functional assays. CHO-K1 cells were incubated with 2 μ Ci/ml of myo-[³H]inositol for 18 h. Thereafter, cells were washed once with serum-free DMEM containing 10 mM LiCl. Accumulation of intracellular inositol phosphate and cAMP were determined as described previously [Grosse et al., 2000].

GST-Rhotekin Fusion Protein

Cultures of *Escherichia coli* (DH5 α) transformed with the plasmid pGEX-rhotekin were grown to an A₆₀₀ of 0.5, and fusion protein expression was induced by addition of 0.3 mM isopropyl-1-thio- β -D-galactoside (Sigma-Aldrich). After 3 h, cells were collected by centrifugation, and the pellet was resuspended in ice-cold TBS containing 5 mM MgCl₂, 1 mM DTT, and 5 mM phenylmethylsulfonyl fluoride. After sonification of lysates, Triton X-100 was added to a final concentration of 1%, and lysates were incubated at 4°C for 30 min. Lysates were then centrifuged (12,000g, 10 min at 4°C), and glycerol was added to a final concentration of 10%. The fusion protein preparations were stored in aliquots at -80°C for up to 8 weeks.

Determination of Rho Activity

Activation of Rho was measured as previously described with minor modifications [Ren et al., 1999]. After stimulation of CHO-K1 cells stably transfected with RC/CMV vector (Invitrogen) or the human S1P₄-myc construct in 6 cm dishes, monolayers were washed with ice-cold TBS and lysed for 15 min by the addition of 300 μ l of Rho-RIPA buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1% Triton X-100, 0.1 μ M aprotinin, 1 μ M

leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Lysates were cleared by centrifugation and incubated for 30 min at 4°C with GST-rhotekin precoupled to glutathione-Sepharose 4B (Amersham Pharmacia Biotech). Sepharose beads were collected by centrifugation (5,000 rpm, 1 min at 4°C), washed three times with RIPA buffer, and proteins were denatured in SDS sample buffer. Precipitates were resolved by SDS-PAGE on 15% acrylamide gels, and proteins were subsequently transferred to Biotrace polyvinylidene difluoride membranes (Pall Gelman Laboratory, Ann Arbor, MI). Rho proteins were probed with the monoclonal Rho antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a secondary horseradish peroxidase-conjugated anti-mouse IgG antiserum (Sigma-Aldrich) and visualized on X-ray film (Eastman Kodak) by enzyme-linked chemiluminescence (Amersham Pharmacia Biotech).

Cell Staining

CHO-K1 cells were seeded on coverslips with about 10% confluence 1 day before they starved serum-free for 16 h. They were washed three times with PBS-d (Gibco/BRL) and fixed by adding 5% paraformaldehyde (Sigma-Aldrich) for 15 min on ice. After three additional washing steps and permeabilization with 0.5% Triton-X-100 (Merck) for 5 min at room temperature, cells were incubated with 5% milk powder for 1 h. To investigate cytoskeleton rearrangements, Alexa-fluor-488 labeled phalloidin (Molecular Probes, Eugene, OR) was added for 1 h. For visualization of hS1P₄-myc expression, cells were incubated for 1 h with the murine anti-*myc*-epitope antibody 9E10 (Roche Molecular Biochemicals, Indianapolis, IN). After three washing steps with PBS-d, cells were incubated for 1 h with donkey-anti-mouse-Cy3 antibodies (Jackson Immunoresearch Laboratories). Cells were analyzed by fluorescence microscopy as described previously [Kusch et al., 2000].

Chemotaxis Assays

Transwell chambers (6.5 mm diameter, pores with 5 µm diameter, Costar) were incubated in 20 µg/ml mouse collagen type IV (BD Biosciences, San Diego, CA) at 4°C overnight and washed two times with PBS-d. Jurkat cells were grown overnight in RPMI medium supplemented with 2 mM L-glutamine and 1% FCS. After

resuspending the cells in RPMI containing the appropriate stimulus, 2 mM L-glutamine, 25 mM HEPES, pH 7.3, and 0.1% fatty acid-free bovine serum albumin (Sigma-Aldrich), 100 µl of the cell suspension (1×10^7 cells/ml) was added to the upper chamber. Four hundred and fifty microlitres of the same medium was used for the lower chamber. Cells were incubated for 4 h under normal cell culture conditions. Subsequently, 350 µl of the lower chamber was concentrated to 50 µl, and cells were counted for 1 min by flow cytometry (BD Biosciences).

Receptor Internalization Experiments

After serum-free starvation for 16 h prior to analysis, cells were briefly trypsinized and resuspended in DMEM containing 25 mM HEPES and 0.1% fatty acid-free bovine serum albumin. The appropriate stimulus was added, and the cells were incubated at 37°C. After the corresponding time, 100 µl of the cell suspension was added to 1.4 ml of ice-cold FACS-buffer (PBS-d, 4% FCS, 10 mM EDTA, pH 7.3). Cells were kept on ice and analyzed by FACS.

Generation of Monoclonal Antibodies Against the Murine S1P₄ Receptor

The N-terminus (aa 2-56) of the murine S1P₄ receptor (mS1P₄) was expressed as a C-terminal hexahistidine fusion protein using the pQE60 vector system (Qiagen, Valencia, CA) according to the manufacturer's protocol under denaturing conditions. Immunization of rats and the generation of hybridoma cells was done according to standard protocols. Thirty four different hybridoma supernatants from one fusion were positive in a sandwich-ELISA using the same hexahistidine fusion protein that was also used for immunization. Five of these supernatants were also positive in a FACS-analysis with transiently mS1P₄ transfected CHO-K1 cells, three of which (2A1, 6D7, and 8B4) could be established by limiting dilution and grown as single clones. The antibodies of all three supernatants match the IgG 2Ak immunoglobulin subtype as determined by standard protocols.

RESULTS

Generation of Cell Lines Stably-Expressing S1P₄

Since many cultured cells respond to an S1P challenge through endogenous S1P receptors, it was necessary to establish a heterologous

expression system with low background activity in order to elucidate the specific signaling pathways triggered by the human S1P₄ receptor (hS1P₄). Previous studies have already demonstrated that CHO-K1 cells give a low background response upon stimulation with S1P [Kon et al., 1999], and PCR-studies of Jurkat cells showed only a low amount of S1P₄-template present in these cells [Motohashi et al., 2000]. CHO-K1 and Jurkat cell lines stably expressing hS1P₄ were generated with a C-terminal myc-epitope tag (CHO-K1/hS1P₄-myc and Jurkat/hS1P₄-myc). Both transfected cell lines were tested for S1P₄ expression by FACS analysis (Fig. 1A). Surface expression of S1P₄ on stably transfected CHO-K1 cells was also confirmed by confocal microscopy (Fig. 1B). Both cell lines were used to determine the

functional characteristics of S1P₄ with regard to its signaling properties and with reference to S1P₄ mediated cellular responses.

G-Protein-Coupling of the S1P₄ Receptor

First we examined the direct coupling of G α -subunits of trimeric G-proteins to the S1P₄ receptor. For this purpose, membrane preparations of CHO-K1/hS1P₄-myc cells were incubated with the GTP analog [α -³²P]GTP azidoanilide. Following stimulation with S1P, [α -³²P]GTP azidoanilide binds to the corresponding activated G α -subunits but cannot be hydrolyzed efficiently. Therefore, activated G α -subunits bound to [α -³²P]GTP azidoanilide accumulate and can be selectively detected on an autoradiogram after immunoprecipitation with specific anti-G α antisera and subsequent SDS-PAGE. Antisera directed against G $\alpha_{15/16}$, G $\alpha_{12/13}$, G α_q , and G α_i were used to determine the specific G α -coupling of S1P₄. These studies revealed that S1P₄ activates G α_i and G $\alpha_{12/13}$ -subunits of trimeric G-proteins (Fig. 2A,B). G α_q is not affected (Fig. 2A,B). In particular, the G $\alpha_{12/13}$ -subunits are activated very potently. G $\alpha_{15/16}$ which has recently been suggested to be linked to S1P₄ because of their genetic proximity [Contos et al., 2002] is not activated by S1P₄. As a control CHO-K1 cells stably transfected with gonadotropin-releasing hormone (GnRH) receptor [Grosse et al., 2000] did not activate any of the G α -subunits examined after stimulation with S1P (Fig. 2A,B). The specificity of the antisera and the expression of the tested G α -subunits in CHO cells have already been demonstrated [Grosse et al., 2000].

Effect of S1P₄ on PLC Activation

S1P₄ activates the MAP kinase Erk1/2 [Van Brocklyn et al., 2000]. This signaling pathway is Ptx-sensitive and therefore dependent on the activation of G α_i -subunits of trimeric G-proteins. To examine other downstream signaling properties of S1P₄ linked to G-proteins, we assessed activation of the PLC by determining inositol phosphate accumulation in CHO-K1/hS1P₄-myc cells. As shown in Figure 3A, more than a twofold increase in PLC activation was observed after stimulation with 1 μ M S1P in S1P₄ transfected CHO-K1 cells, whereas only a marginal response was detected in untransfected control cells. The S1P₄ mediated PLC activation is Ptx-sensitive and therefore dependent on G α_i activation. In addition, cells were

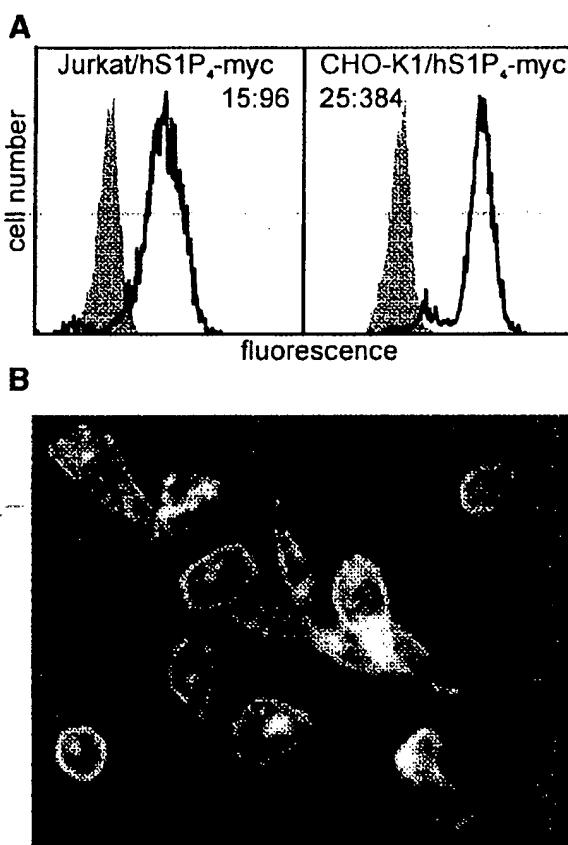


Fig. 1. Stable expression of hS1P₄-myc on CHO-K1 and Jurkat cell lines. **A:** FACS-analysis of stably hS1P₄-myc transfected CHO-K1 and Jurkat cell lines. Cells were fixed and permeabilized prior to detection of the intracellular C-terminal myc-epitope tag with the specific monoclonal anti-myc-epitope antibody 9E10. Vector transfected control cells are shown in gray, and linear means are given by numbers. **B:** Surface expression of hS1P₄-myc on transfected CHO-K1 cells, detected with the anti-myc-epitope antibody 9E10.

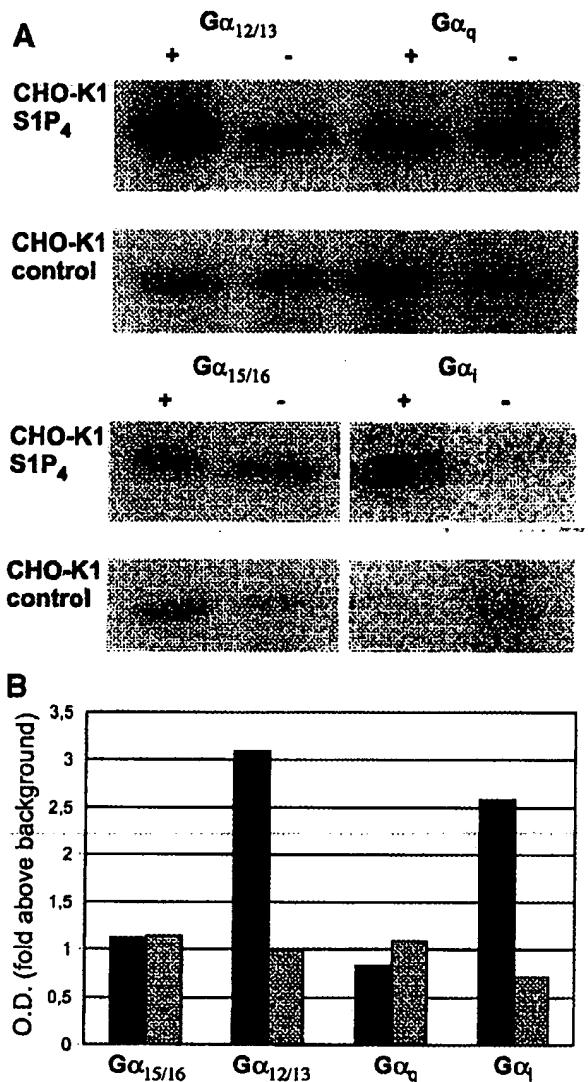


Fig. 2. Activated G-proteins in membrane preparations of CHO-K1/hS1P₄-myc cells. Membrane preparations were photo-labeled with [α -³²P]GTP azidoanilide in the absence (-) or presence (+) of 1 μ M S1P and immunoprecipitated with the G α _{15/16}, G α _{12/13}, G α _q, and G α _i antisera as described in "Materials and Methods." A: Precipitated proteins were resolved in SDS-PAGE and visualized by autoradiography. B: Densitometric analysis of the corresponding signals on the autoradiogram normalized towards the expression in control cells. S1P-stimulated cell preparations are shown in black, unstimulated controls are shown in gray.

stimulated with 1 mM ATP. The corresponding ATP receptors couple to G α _{q/11}-subunits of trimeric G-proteins that are much more potent in activating PLC than the $\beta\gamma$ -subunits responsible for the G α _i-induced PLC activation resulting in an up to fivefold increase in inositol phosphate production (Fig. 3A). The ATP-induced PLC activation is Ptx-insensitive and

therefore not linked to G α _i-coupled signaling pathways (Fig. 3A).

S1P₄ Does not Induce cAMP Accumulation

The adenylyl cyclase is known to be an important effector of trimeric G-proteins mainly activated by G α _s subunits. Therefore, we were interested whether stimulation of S1P₄ would lead to an increase of the intracellular cAMP level or not. For this purpose, we stimulated CHO-K1/hS1P₄-myc cells and vector transfected control cells with 1 μ M S1P for 1 h and tested for cAMP accumulation as described in

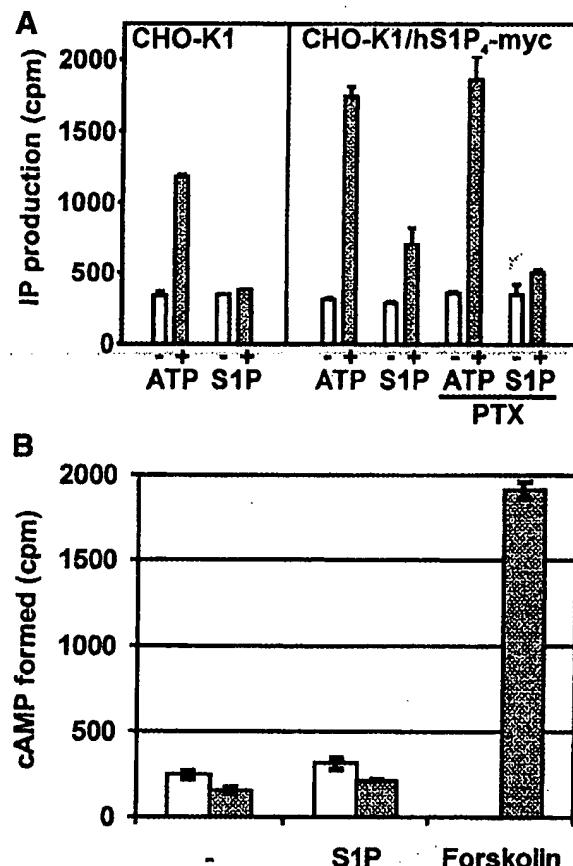


Fig. 3. Second messenger production in CHO-K1/hS1P₄-myc cells. A: Ptx-sensitive inositol phosphate accumulation induced by the S1P₄ receptor. Non-transfected CHO-K1 cells and CHO-K1/hS1P₄-myc cells were seeded in 12-well plates and incubated in the presence of Ptx (0.1 μ g/ml, 18 h prior to stimulation) as indicated. Inositol phosphate accumulation was determined after 45 min of incubation with vehicle (-), 1 μ M S1P, and 1 mM ATP (+) respectively. Data are means \pm SE of two independent experiments, each performed in triplicate. B: cAMP formation in vector transfected (white) versus hS1P₄-myc (gray) expressing CHO-K1 cells. Cells were incubated with 1 μ M S1P or 50 μ M forskolin for 1 h as indicated. Shown are means \pm SE of triplicates.

"Materials and Methods." The hS1P₄ expressing CHO-K1 cells as well as the control cells show a slight increase in cAMP production upon S1P stimulation, but no S1P₄ specific effect could be detected (Fig. 3B). Forskolin as a positive control directly activates the adenylyl cyclase and produced more than a tenfold increase in cAMP accumulation in CHO-K1/hS1P₄-myc cells (Fig. 3B). Thus, S1P₄ does not activate the adenylyl cyclase via the G α_s subunit of trimeric G-proteins.

S1P₄ Mediated Effect on Cell Motility

The migration of lymphocytes, macrophages, and dendritic cells determines their correct trafficking and homing. By examining the migratory capacity of S1P₄ overexpressing cells, an enhanced motility of Jurkat/hS1P₄-myc cells was observed even in the absence of S1P (Fig. 4). S1P₄ receptor-mediated cell motility was sensitive to Ptx, indicating that this effect is linked to the G α_i -subunit of trimeric G-proteins (Fig. 4). Interestingly, primary mouse splenocytes also show a reduced cell motility after Ptx treatment (Fig. 4). S1P₄ is highly expressed on these cells [Gräler et al., 2002], suggesting that S1P₄ induces the observed increase in cell motility

also on primary lymphocytes. Vector transfected control cells did not exhibit an increased motility (Fig. 4). Jurkat/hS1P₄-myc cells as well as control cells show a 1.5-fold increase of cell motility in the presence of 10 nM S1P (Fig. 4). This effect is barely seen on primary mouse splenocytes and not dependent on hS1P₄-myc expression (Fig. 4), indicating that it is not linked to S1P₄-induced signaling pathways. S1P did not elicit migration of Jurkat/hS1P₄-myc cells compared with vector transfected control cells (Fig. 4). In addition, we did not detect migration of CHO-K1/hS1P₄-myc cells using S1P concentrations between 0.1 nM and 1 μ M. Furthermore, expression and stimulation of S1P₄ did not affect the migratory capacity of Jurkat cells endogenously expressing the CXCR4 chemokine receptor towards its specific ligand SDF-1 (data not shown).

S1P₄ Regulates Rho Activation

The small GTPase Rho can be activated by a specific subgroup of trimeric G-proteins and is involved in several cellular responses like cytoskeleton rearrangements, integrin-mediated adhesion, and actin-dependent effector responses. We performed a Rho-assay to examine the

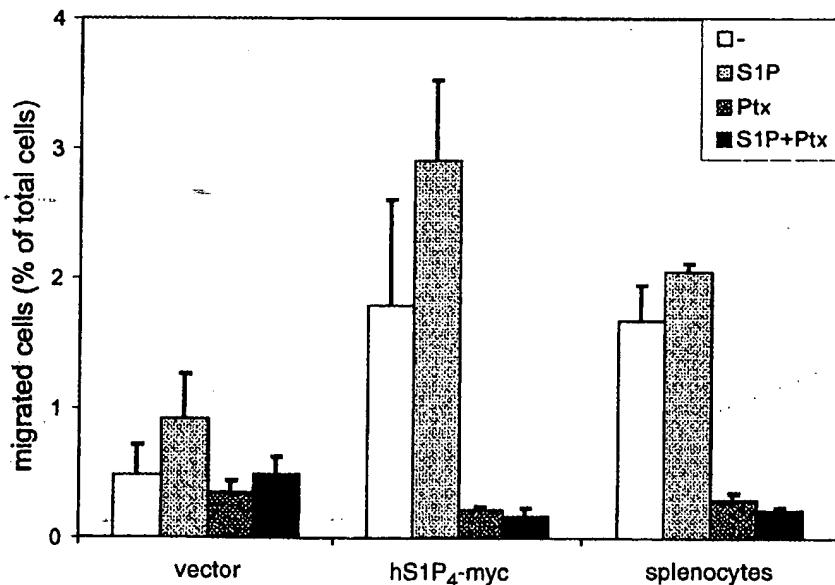


Fig. 4. Increased agonist-independent Ptx-sensitive cell motility of stably hS1P₄-myc transfected Jurkat cells. Stably vector transfected or stably hS1P₄-myc transfected Jurkat cells as well as freshly isolated mouse splenocytes were seeded in Transwell-chambers with or without simultaneous S1P stimulus (10 nM) in the upper and in the lower chamber as described in "Materials and Methods." Cells were incubated with 0.1 μ g/ml Ptx for 2 h prior to the experiment when indicated. After 4 h, cells were harvested and counted. Shown are means \pm SE, $n \geq 4$.

regulatory capacity of S1P₄ on Rho activation. Whereas vector transfected control cells do not show any significant increase in Rho-activation upon S1P stimulation, CHO-K1/hS1P₄-myc cells rapidly activate Rho within 1 min after S1P stimulation (Fig. 5A,B). The activity of Rho declines within 20 min after S1P stimulation, indicating the short half-life of S1P₄-induced Rho activation. Thus, S1P₄ is a very rapid and potent activator of the small GTPase Rho.

S1P₄ Mediated Cytoskeleton Rearrangements

An important event with respect to changes in adhesion and motility of cells are alterations within their cytoskeleton to promote a higher degree of mobility for managing new circumstances. To examine the influence of S1P₄ in

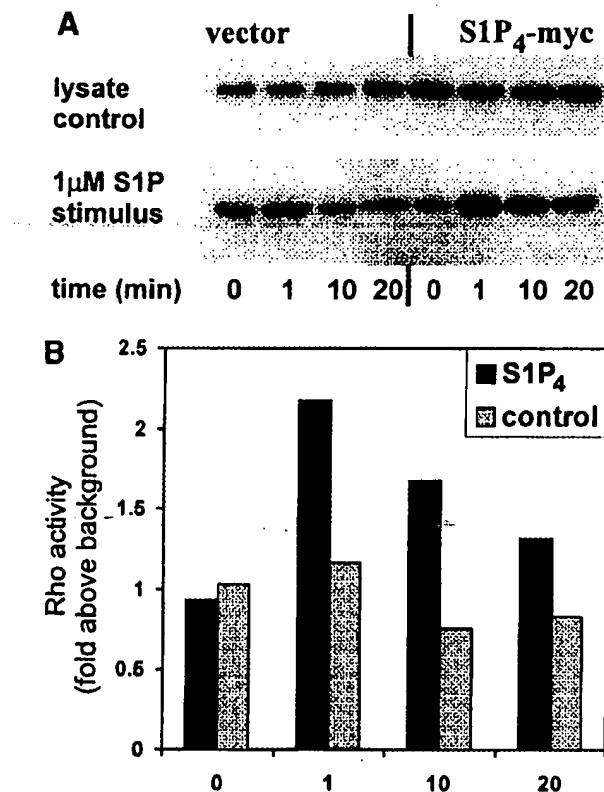


Fig. 5. Activation of Rho in CHO-K1/hS1P₄-myc cells. **A:** Vector transfected CHO-K1 cells and CHO-K1/hS1P₄-myc cells starved with 0.5% serum overnight prior to stimulation with 1 μ M S1P for 1, 10, and 20 min. GTP-bound RhoA was analyzed applying a pull down assay using SEPHAROSE conjugated Rhotein-GST fusion protein. The lysate control shows equal amounts of Rho expressed in each cell preparation. Shown is one representative out of two similar results. **B:** Densitometric quantification of the signals shown in (A). Vector transfected cells are shown in gray, hS1P₄-myc transfected cells are shown in black.

this connection, we looked for S1P-induced cytoskeleton rearrangements in stably transfected CHO-K1 cells. No significant increase in the amount of peripheral stress fibers and cell rounding can be seen in control cells (Fig. 6A). Addition of S1P to CHO-K1/hS1P₄-myc cells however induces a significant increase in the amount of peripheral stress fibers and rounded cells (Fig. 6B). The amount of rounded cells in the total cell population as determined by three independent cell counts was 3 \pm 1% SE for unstimulated CHO-K1 control cells and CHO-K1/hS1P₄-myc cells, 5 \pm 2% SE for S1P-stimulated

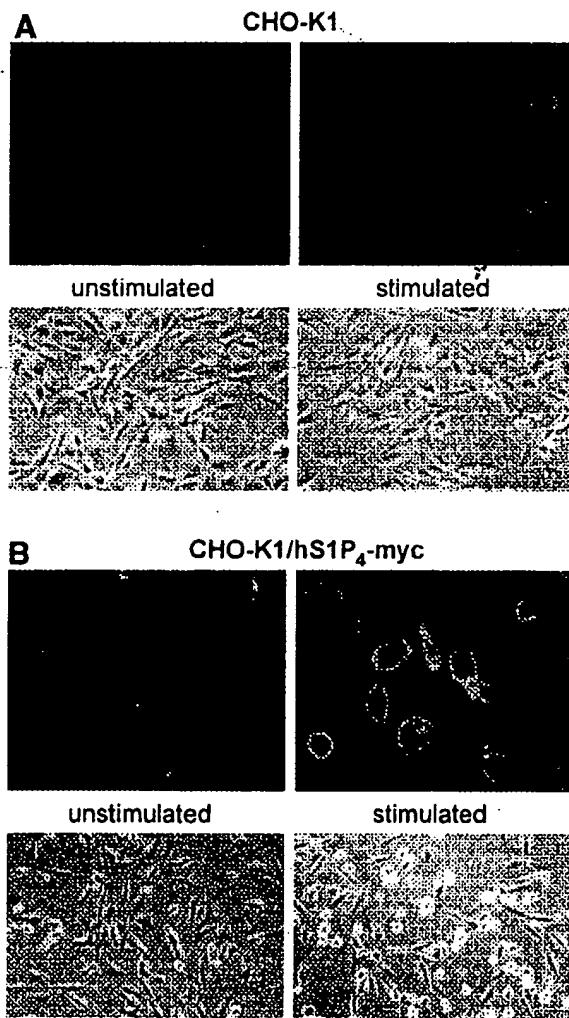


Fig. 6. Cytoskeleton rearrangements in CHO-K1/hS1P₄-myc cells. **A:** Non-transfected CHO-K1 cells and **(B)** CHO-K1/hS1P₄-myc cells were seeded on cover-slips in six-well plates, starved serum-free for 16 h, and were subsequently stimulated for 20 min with 1 μ M S1P as indicated. In the upper row cells were stained with Alexa-fluor-488 phalloidin and analyzed by confocal microscopy, in the lower row the cell shape is shown by light microscopy.

control cells, and $53 \pm 7\%$ SE for S1P-stimulated CHO-K1/hS1P₄-myc cells.

Agonist-Induced Internalization of S1P₄

An important aspect of functional studies is the correct expression and presentation of the receptor on the cell surface. Using the hS1P₄-myc construct we provided already in an earlier study evidence that hS1P₄ is inserted into the plasma membrane on the cell surface in the expected orientation with the N-terminus facing the cells exterior and the C-terminus inside the cell [Van Brocklyn et al., 2000]. For the purpose of further functional studies we generated monoclonal antibodies against murine S1P₄ (mS1P₄, see "Materials and Methods"). These antibodies are suitable to detect mS1P₄-myc, but not hS1P₄-myc on the surface of transiently transfected HEK293 cells by flow cytometry (Fig. 7A). Western-blots of membrane protein extracts from these cells revealed a specific signal around 42 kDa that was not found on Interleucin-8 receptor A transfected HEK293 cells (Fig. 7B). A second weaker signal appears at about 46 kDa (Fig. 7B). Since there is no need of cell permeabilization in order to recognize the antibody epitope we used these

antibodies to monitor the kinetics of mS1P₄ surface expression upon addition of S1P. Within minutes after stimulation with 1 μ M S1P, the mS1P₄ specific signal on these cells significantly decreases and reaches a minimum of approximately 65% compared to the unstimulated control cells after 45 min (Fig. 7C). This effect is specific for S1P and cannot be seen with other stimuli like LPA (Fig. 7C). However, Ptx does not interfere with receptor internalization, and our monoclonal antibodies against mS1P₄ are unable to trigger the internalization process (data not shown). Cells that were permeabilized after S1P stimulation for the specified time points show the same receptor signal as unstimulated cells (data not shown). This demonstrates that the complete amount of detected mS1P₄ does not change. Therefore, mS1P₄ receptors are not degraded, and S1P binding to mS1P₄ does not interfere with antibody binding.

DISCUSSION

The responses of diverse target cells to low molecular weight lipid compounds, such as S1P and LPA have been shown to be mediated in large part by G protein-coupled receptors which bind these lipids and thus mediate their physiological functions. We have cloned recently one member of the S1P receptor family, S1P₄, and showed that it specifically binds S1P as an agonist [Gräler et al., 1998; Van Brocklyn et al., 2000]. However, apart from its obvious field of activity, the immune system, little is known about the importance and physiology of S1P₄ in this distinct compartment. This report provides information regarding the signal cascade and cellular responses triggered by the S1P₄-S1P interaction.

One of the first events with regard to GPCR signaling is the activation of certain G-proteins. We demonstrate in this study that S1P₄ activates the G-protein subunits G α_i and G $\alpha_{12/13}$ by two different methods: (1) By direct labeling of the activated G α -subunits and (2) by analyzing functional downstream signaling events. S1P₄ activates G $\alpha_{12/13}$ very potently without affecting G α_q (Fig. 2). Other S1P receptors such as S1P₂ and S1P₃ couple with both, G $\alpha_{12/13}$ and G α_q respectively [Windh et al., 1999]. All S1P receptors currently known signal via the G α_i -subunit of trimeric G-proteins [An et al., 1998b; Windh et al., 1999]. Thus, S1P₄ differs from these S1P

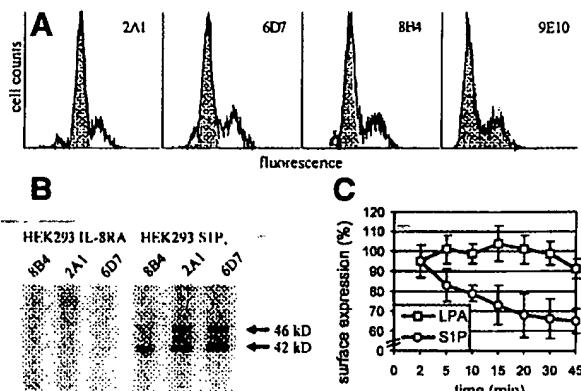


Fig. 7. Detection of the murine S1P₄ receptor on transiently transfected HEK293 cells. **A:** FACS-analysis of anti-mouse-S1P₄ monoclonal antibodies 2A1, 6D7, and 8B4 on mouse S1P₄-myc (black line) and on human S1P₄-myc (filled gray) transfected HEK293 cells. The anti-myc-epitope antibody 9E10 is shown as a control. **B:** Western-blot with membrane preparations of Interleucin-8 receptor A and mouse S1P₄-myc transfected HEK293 cells. **C:** Detection of mS1P₄-myc on the surface of transiently transfected HEK293 cells using the anti-murine S1P₄ antibodies 8B4, 2A1, and 6D7 by FACS-analysis. After addition of 1 μ M S1P, the murine S1P₄ surface expression decreases within 45 min (circles). One micromolar LPA has no significant influence on the surface expression of S1P₄ (rectangles). The FACS signal was correlated to unstimulated cells at the corresponding time point. Shown are means \pm SE, $n \geq 3$.

receptors concerning its selective G protein-coupling. S1P₄ is also not inducing an increase in cAMP accumulation (Fig. 3B). Therefore, it is unlikely that S1P₄ couples to the G α_s -subunit of trimeric G proteins. Other S1P receptors like S1P₂ and S1P₃ are able to activate the adenylyl cyclase very potently resulting in cAMP levels up to 10 times higher than vector controls [Kon et al., 1999] although direct coupling studies performed with [³⁵S]GTP γ S did not show any specific G α_s -coupling [Windh et al., 1999]. A recent study suggests that S1P₄ is linked to G $\alpha_{15/16}$ signaling because of their genomic proximity, a fact that we already published in 1999 [Gräler et al., 1999]. This hypothesis does not stand a closer look because G $\alpha_{15/16}$ does not couple to S1P₄ (Fig. 2A,B), and the human transcript for G α_{16} does not show the same expression pattern as S1P₄ (data not shown).

Some downstream signaling events induced by S1P₄ like the activation of the mitogen-activated protein-kinases (MAP kinases) ERK1/2, the activation of PLC, or the ability to open intracellular calcium stores have already been investigated [Van Brocklyn et al., 2000; Yamazaki et al., 2000]. We confirmed the S1P₄-induced Ptx-sensitive PLC activation in our CHO-K1 cell system as a functional G α_i -coupled signaling pathway (Fig. 3A). Our results concerning S1P₄-induced Rho activation (Fig. 5) and cytoskeleton rearrangements (Fig. 6) form a link to the G $\alpha_{12/13}$ directed signal transduction because former studies have shown that stress fiber formation is coupled via Rho to G $\alpha_{12/13}$ activation [Buhl et al., 1995]. G α_{13} is able to directly stimulate the p115 RhoGEF-catalysed guanine nucleotide exchange on Rho [Hart et al., 1998]. In addition, the described effects on cell rounding and peripheral stress fiber formation suggest an involvement of S1P₄ on the cell shape that could be important for its *in vivo* function (Fig. 6B). Similar results concerning cell rounding obtained from studies of S1P₂ and S1P₃ overexpressing HEK293 and pheochromocytoma PC12 rat cells [Van Brocklyn et al., 1999] as well as from S1P₂ overexpressing CHO cells with respect to stress fiber formation [Gonda et al., 1999] may be taken as an indication for a physiological feature commonly induced by S1P receptors.

It may be surprising that the ectopic expression of S1P₄ in Jurkat cells (Fig. 3) per se is already sufficient to direct cells towards a phenotype observed later on when its ligand

S1P is added. This could be interpreted to mean that S1P₄ has a high intrinsic activity in terms of some independence from ligand binding for signaling or that T cells produce S1P at a level sufficient for signaling. However, this observation may well be a consequence of the ubiquitous presence of low amounts of ligand produced by the cells thus initiating autocrine stimulation. This renders the study of S1P receptors inevitably complicated because it is difficult to establish suitable cell-based test systems. Given the widespread distribution of S1P receptors and their ligand S1P in tissues it is worth speculating that an apparently unstimulated stage of an observed cell should be more precisely defined as a low level-induced stage, at least from the point of view of S1P-based cell physiology. In keeping with this hypothesis, other reports have shown that LPA₂ is able to activate G α_{i1} in the baculovirus expression system in the absence of the appropriate ligand [Yoshida and Ueda, 1999]. Moreover S1P₂ and S1P₃ induce cell rounding in pheochromocytoma PC12 rat cells in a ligand-independent fashion [Van Brocklyn et al., 1999]. Therefore, a ligand-independent basal activity could not only be observed with S1P₄, but also with other LPA and S1P receptors like LPA₂, S1P₂, and S1P₃, indicating that the mere expression of these receptors can significantly activate the cells even in the absence of the specific ligand. In this connection, it could be important for the cell to regulate S1P receptor trafficking as an additional control for their signaling capacities and for S1P receptor selectivity. As already shown for S1P₁ [Lee et al., 1998; Liu et al., 1999], S1P₄ undergoes ligand-induced trafficking. Surface expression of S1P₄ on HEK293 cells decreases within minutes after S1P stimulation (Fig. 7C), indicating that the receptor undergoes ligand-induced internalization or sequestration. This process is supposed to be an important step in receptor resensitization rather than desensitization after activation of the receptor [Ferguson and Caron, 1998]. The finding of an incomplete receptor clearance may be explained in part by the onset of counteracting process, i.e., the recycling of internalized S1P₄ to the cell surface. It may also be important for cells to keep a certain level of S1P₄ receptor expressed on the surface to maintain a constitutive signaling since lymphocytes expressing S1P₄ are constantly exposed to physiological S1P levels in blood [Igarashi and Yatomi, 1998].

The second weaker signal at about 46 kDa in the Western-blot with mS1P₄ transfected HEK293 cells (Fig. 7B) may derive from a different posttranslational receptor processing. A partially more efficient glycosylation of the transfected receptor for example could account for these two separate signals. And it has been shown recently that N-glycosylation of S1P₁ facilitates ligand-induced receptor internalization [Kohno et al., 2002].

With regard to the immune system as the main compartment of S1P₄ expression, it is known that another class of GPCRs, the chemokine receptors, play a pivotal role for the trafficking and homing of lymphocytes [Förster et al., 1996, 1999]. It is conceivable that S1P₄ may also be an important player in this scenario influencing the activation and migration behavior of B- and T-lymphocytes and probably the microenvironment of lymphoid organs. This hypothesis is supported by our finding that overexpression of human S1P₄ in the T-cell line Jurkat resulted in an increased cell motility which can be augmented further by the presence of the ligand S1P (Fig. 4). Remarkably S1P₁ and S1P₃ have already been shown to induce cell migration towards S1P [Kon et al., 1999]. In contrast to S1P₁ and S1P₃, an inhibitory effect on cell migration as well as on Rac activation and membrane ruffling has recently been demonstrated for S1P₂ [Okamoto et al., 2000]. Thus, S1P receptors could be important for the fine tuning of migratory properties of cells leading to an increased or decreased migration and motility respectively. The analysis of S1P₁ deficient mice makes clear that these cellular responses are able to affect critical functions *in vivo* [Liu et al., 2000] suggesting a comparable influence of S1P₄ in immune surveillance.

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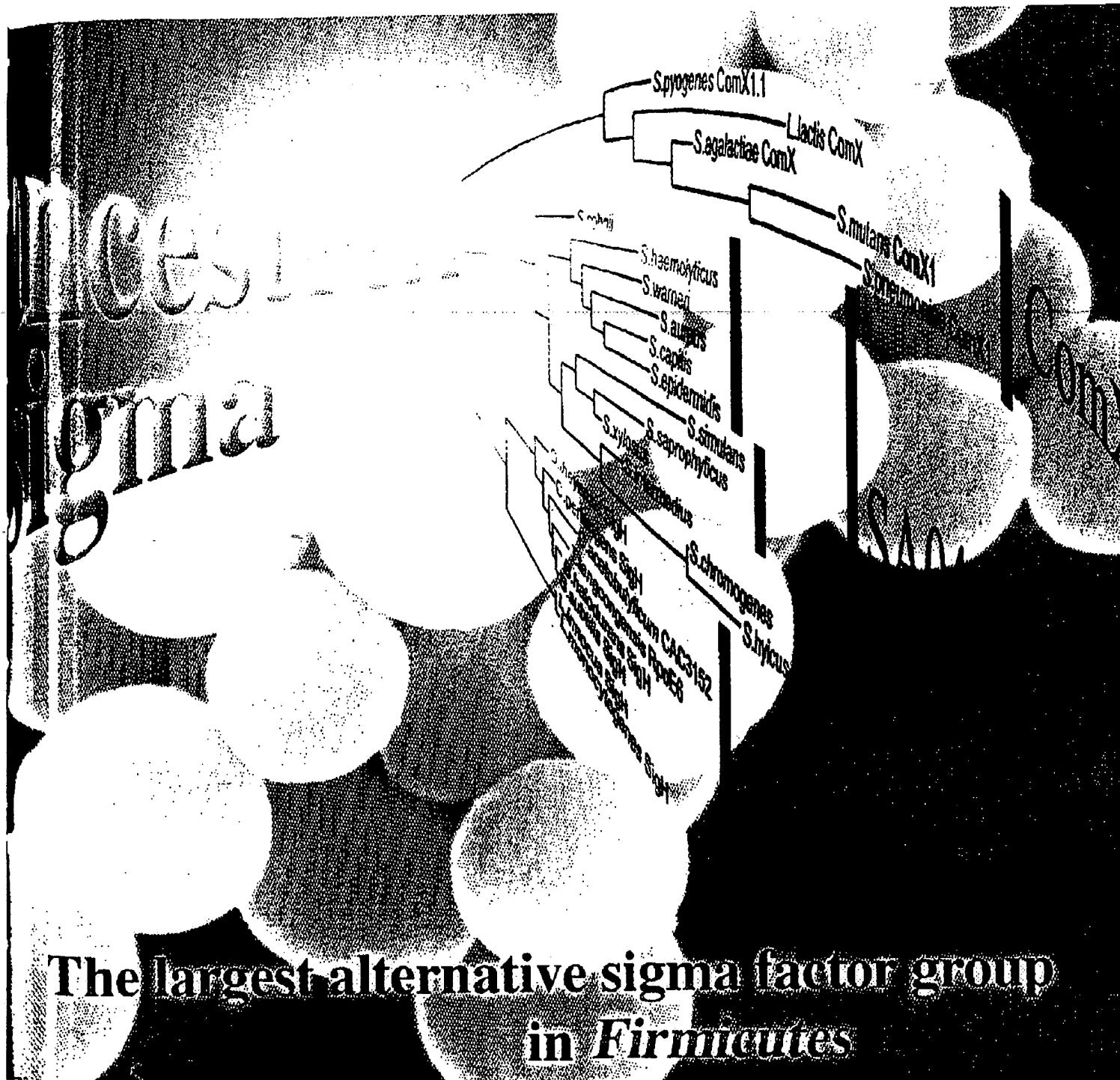
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Sphingosine 1-phosphate promotes cell migration through the activation of Cdc42 in Edg-6/S1P4-expressing cells

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Abstracts

Background: Sphingosine 1-phosphate (Sph-1-P) is a bioactive lipid mediator released from activated platelets, which regulates diverse signal transduction pathways via cell surface receptors. Recent studies have revealed that the seven-transmembrane-spanning receptors, Edg-1, Edg-3, Edg-5, Edg-6 and Edg-8 are specific Sph-1-P receptors. Northern blot analysis has demonstrated that Edg-6 is expressed in lymphocyte-containing tissues such as spleen and lung. Little is known about the molecular mechanisms of Edg-6 functions, probably because of the difficulties in expressing Edg-6 on the cell surface.

Results: Here, our studies revealed that N-terminal

FLAG-tagged Edg-6 or Edg-6-GFP fusion protein was expressed in the endoplasmic reticulum, but was not expressed on the cell surface. On the other hand, C-terminally tagged Edg-6 or both N-terminally and C-terminally tagged Edg-6 was able to localize to the cell surface. Using these cells, we found that Sph-1-P induced cell migration through cell surface-expressed Edg-6 in a pertussis toxin-sensitive manner. This motility was mediated through the activation of a member of the Rho family of small GTPases, Cdc42.

Conclusion: These results support a role for Sph-1-P signalling via Edg-6 in the pathways involved in cell motility.

Introduction

Sphingosine 1-phosphate (Sph-1-P) is a bioactive lipid mediator known to be released from activated platelets (Yatomi *et al.* 1995). To date, five seven-transmembrane-spanning receptors, Edg-1/S1P1, Edg-3/S1P3, Edg-5/S1P2, Edg-6/S1P4 and Edg-8/S1P5, have been identified as specific Sph-1-P receptors (Kluk & Hla 2002). Analysis of Edg-1/S1P1-deficient mice demonstrated that this receptor is essential for vascular maturation via activation of the small GTPase Rac, following Sph-1-P stimulation (Liu *et al.* 2000). Additionally, Edg-3/S1P3 and/or Edg-5/S1P2-deficient mice present a neuronal dysfunction (Ishii *et al.* 2001, 2002; MacLennan *et al.* 2001).

Northern blot analysis has demonstrated that Edg-1/S1P1, Edg-3/S1P3 and Edg-5/S1P2 are expressed ubiquitously in many mammalian tissues (Zhang *et al.* 1999).

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In contrast, Edg-6/S1P4 is specifically expressed in lymphocyte-containing tissues such as spleen and lung (Graler *et al.* 1998), so examining the signalling pathways mediated through this receptor is important in elucidating the effects of Sph-1-P in lymphoid cells. Recent reports have revealed that a phosphorylated form of FTY720, the immunosuppressive agent and a structural analogue of sphingosine, is a potent agonist for Sph-1-P receptors expressed in lymphocytes or endothelial cells (Brinkmann *et al.* 2002; Mandala *et al.* 2002). However, it is still unknown which of the Edg family receptors mediate the sequestration of lymphocytes following FTY720 treatment.

In lymphocytes, regulation of cell migration is an important function. Reportedly, Sph-1-P can regulate cell migration in some cell types. Sph-1-P has been shown to inhibit cell motility and invasiveness of tumour cells (Sadahira *et al.* 1992), as well as PDGF-induced chemotaxis of smooth muscle cells (Bornfeldt *et al.* 1995). These early reports pointed out that Sph-1-P inhibits cytoskeletal rearrangement. However, more

recent studies have indicated that such Sph-1-P-mediated cell migration involves the regulation of the Rho family of small GTPases via the Sph-1-P receptor Edg-5/S1P2 (Okamoto et al. 2000). In human umbilical vein endothelial cells (HUVECs), Sph-1-P also promoted cell motility (Lee et al. 1999). That report revealed that Edg-1/S1P1- or Edg-3/S1P3-mediated signalling cascades were required for endothelial cell morphogenesis into capillary-like formations, again supporting a role for Rho GTPases.

In contrast to the other Edg-family Sph-1-P receptors, little is known about the molecular mechanisms behind Edg-6/S1P4 functions. Using stable transfecants generated in this study to analyse precise mechanisms of signal transduction mediated by Edg-6/S1P4, may enable us to determine roles for Edg-6/S1P4 signalling pathways in cellular processes such as migration.

Results

Sph-1-P induces cell migration through Edg-6/S1P4 expressed on the cell surface

The Sph-1-P receptor Edg-6/S1P4 was identified in 1998 (Graler et al. 1998). Previous reports demonstrated that Sph-1-P-stimulated Edg-6/S1P4 induced MAPK activation (Van Brocklyn et al. 2000) and Ca^{2+} mobilization (Yamazaki et al. 2000) in a pertussis toxin-sensitive manner. However, we were not able to reproduce these results in our model. We considered the possibility that the difficulty in the protein localizing to the cell surface might account for the lack of progress in our functional analysis of Edg-6/S1P4. To determine the cellular localization of Edg-6/S1P4, we prepared Edg-1/S1P1 and Edg-6/S1P4 constructs fused to GFP (green fluorescent protein) at the C-terminal intracellular portion, and transiently transfected these vectors into human Jurkat T cells. As shown in Fig. 1A, Edg-6/S1P4-GFP was localized in the intracellular compartments, whereas most of the Edg-1/S1P1-GFP was expressed at the cell surface. In suspension cells, it was difficult to determine in which intracellular compartments Edg-6/S1P4 was localized, so we transiently transfected these constructs into NIH3T3 fibroblasts. As shown in Fig. 1B, most of the Edg-6/S1P4-GFP in these cells was found in the intracellular membrane compartment and not on the cell surface, whereas most of the Edg-1/S1P1-GFP was expressed at the cell surface.

The N-terminal portion of a seven-transmembrane-spanning receptor is normally located extracellularly. Therefore, we prepared an Edg-6/S1P4-GFP construct fused to a FLAG (DYKDDDDK)-tag at the N-terminal,

extracellular portion. Then, we transfected FLAG-Edg-6/S1P4-GFP or Edg-6/S1P4-GFP into Chinese hamster ovary (CHO) cells. Interestingly, most of the FLAG-Edg-6/S1P4-GFP was expressed at the cell surface (Fig. 1C, upper panels). On the other hand, Edg-6/S1P4-GFP was localized in the ER in studies using an endoplasmic reticulum (ER)-specific probe (Fig. 1C, middle panels). Since the C-terminal GFP-tagged Edg-6/S1P4 has a larger molecular weight (42 kDa + 27 kDa) than Edg-6/S1P4 itself, the GFP might have affected the cellular distribution. Therefore, we designed another Edg-6/S1P4 fused with an HA (YPYDVPDYA)-tag at the N- or C-terminus. These expression vectors were transiently transfected into CHO cells. As shown in Fig. 1D, C-terminal HA-tagged Edg-6/S1P4 was expressed at the cell surface (upper panels), whereas N-terminal HA-tagged Edg-6/S1P4 was localized at the ER (lower panels). Similar results were obtained when a FLAG-tag was fused with Edg-6/S1P4 at the N- or C-terminus (data not shown).

The regulation of cell motility is important when regarding functions in lymphocytes, one of the few cell types to express Edg-6/S1P4. We examined whether Sph-1-P might regulate cell migration in the Edg-6/S1P4-expressing CHO transfecants. In order to confirm that the rate of cell migration at basal conditions was equivalent between the vector- or Edg-6/S1P4-expressing transfecants, first, we selected the optimal chemoattractants for these cells. Only IGF-I (insulin like growth factor I) and insulin strongly induced cell migration (data not shown), similar to results from another study (Okamoto et al. 2000). Next, we examined the cell motility using CHO cells which transiently coexpressed both Edg-6/S1P4 and GFP. As shown in Fig. 1E, C-terminal HA-tagged Edg-6/S1P4-expressing cells exhibited enhanced cell migration upon Sph-1-P stimulation (upper). On the other hand, because little of the N-terminal HA-tagged Edg-6/S1P4 was expressed at the cell surface (Fig. 1D), only a small number of migrated cells were observed (middle). These results suggest that ligand-induced Edg-6/S1P4 signalling is involved in cell migration in these CHO transfecants.

Sph-1-P induces enhanced cell migration via Edg-6/S1P4 expressed on the cell surface in CHO stable transfecants

We generated stable Edg-6/S1P4 transfecants in CHO cells to analyse the precise receptor functions of Edg-6/S1P4. To identify the expression of full length Edg-6/S1P4 in these cells easily, a FLAG tag was introduced at the extracellular N-terminal portion of the C-terminal

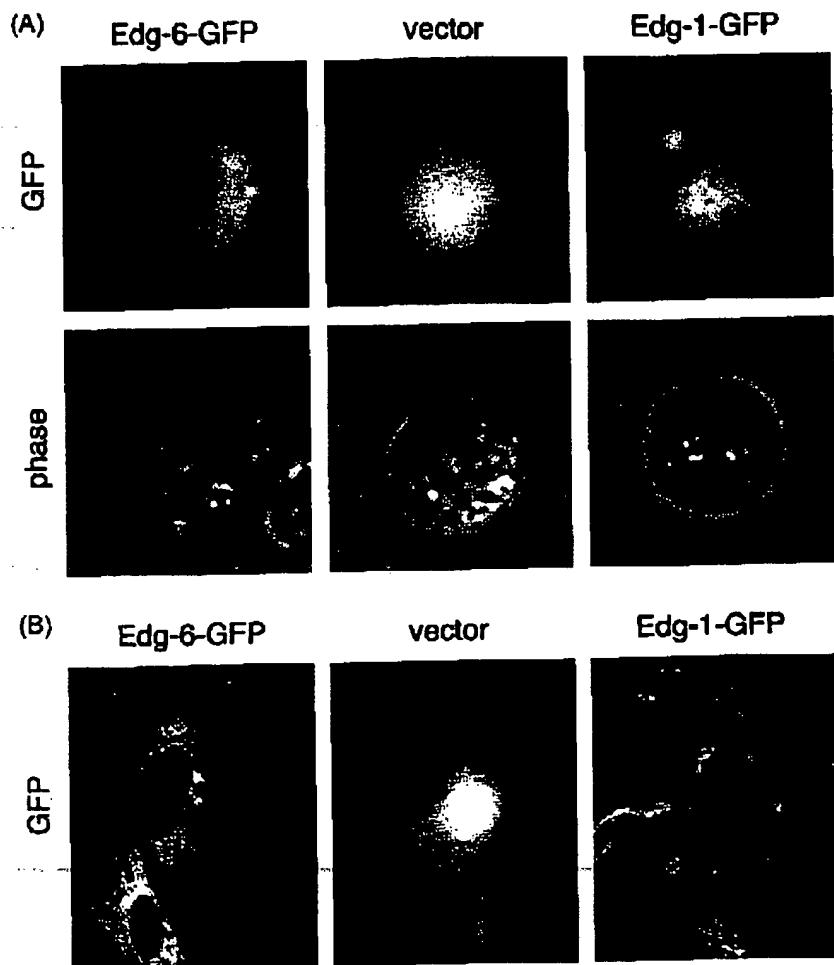


Figure 1 Sph-1-P induces cell migration through Edg-6/S1P4 expressed on the cell surface. Initially, Edg-6/S1P4 had difficulty localizing to the cell surface. Jurkat (A) or NIH3T3 cells (B) were transiently transfected with Edg-1/S1P 1-GFP, Edg-6/S1P4-GFP or GFP alone using Effectene reagent (A) or Lipofect AMINE plus reagent (B). After transfection for 24 h, the expression of GFP fusion proteins was observed in living (A) or fixed (B) cells by fluorescence microscopy. The lower panels in (A) are phase contrast images of the cells. (C) CHO cells were transiently transfected with FLAG-Edg-6/S1P 4-GFP, Edg-6/S1P4-GFP or GFP alone, using Lipofect AMINE plus reagent. F-actin was stained by Alexa 594-phalloidin, and the ER was stained by ER-Tracker Blue-White DPX. The right panels illustrate the merged image of the left three panels, GFP; green, F-actin; red, ER; blue. The arrowhead indicates the edge of the cell surface. (D) CHO cells, transiently transfected with C-terminal HA-Edg-6/S1P4 (upper panels) or N-terminal HA-Edg-6/S1P4 (lower panels), were fixed and permeabilized, then immunostained with an anti-HA antibody (left panels). F-actin was stained by Alexa 594-phalloidin (middle panels). The right panels illustrate the merged image of the left two panels, green; HA, red; F-actin. The arrowhead indicates the edge of the plasma membrane. The asterisks indicate nuclei. (E) CHO cells (2×10^5), transiently co-transfected with GFP and C-terminal HA-Edg-6/S1P4 (upper), or N-terminal HA-Edg-6/S1P4 (middle), or pCDNA3 (lower), were added to the upper well of a Transwell chamber, and 1 μ M Sph-1-P and/or 10 ng/mL IGF-I was placed into the lower chamber, followed by incubation for 4 h at 37 °C. Transmigrated cells on the lower filter were fixed with cold methanol. The number of GFP-positive cells migrating to the lower surface was determined by counting the number of cells in five random fields. These data represent the average of three independent experiments with error bars indicating the SD. Statistical significance: * $P < 0.001$ vs. control.

HA-tagged Edg-6/S1P4 described in Fig. 1D. Immunostaining of these transfectants using anti-FLAG and anti-HA antibodies indicated that most of the Edg-6/S1P4 localized to the cell surface (data not shown). To

further examine the expression of Edg-6/S1P4, intracellular and cell-surface proteins in these cells were separated after cell surface biotinylation and purification with streptavidin-agarose beads. These samples were

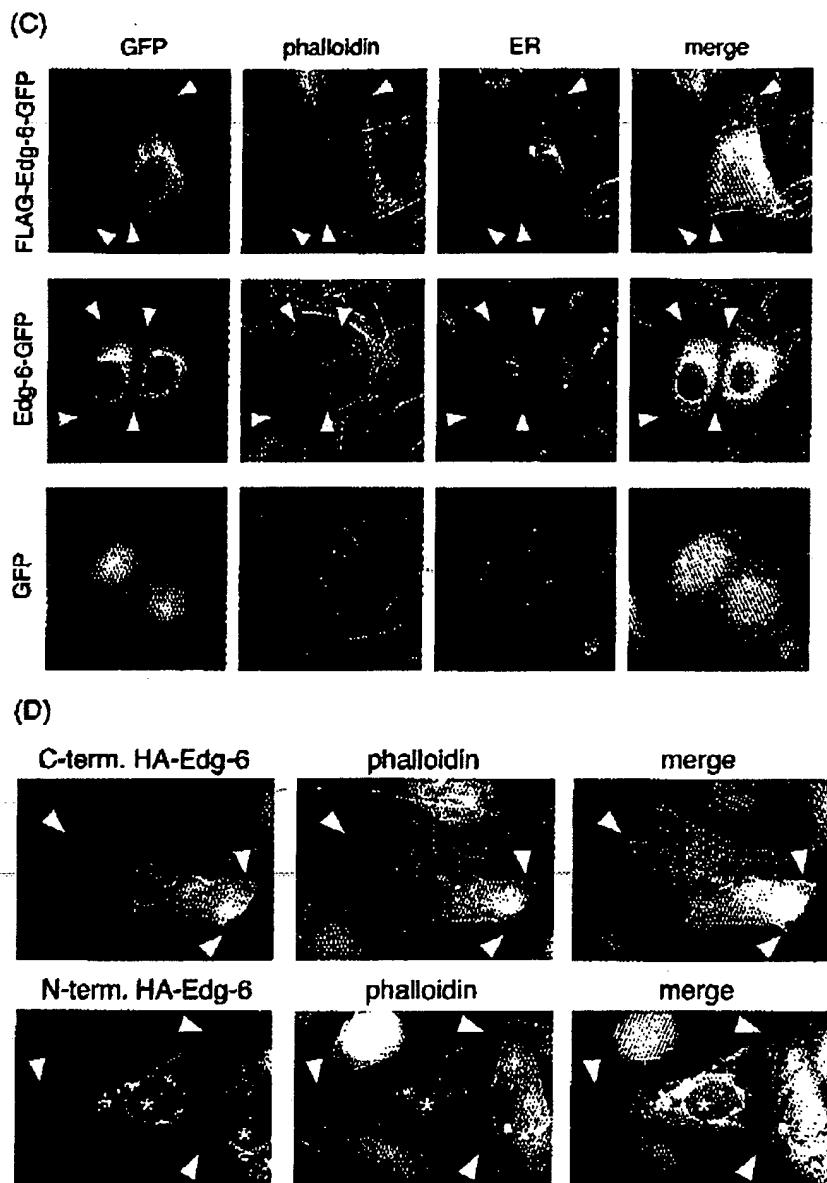


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separated by SDS-PAGE and then analysed by Western blot using an anti-MAPK antibody, to confirm the contamination of intracellular proteins into the cell surface fraction (Fig. 2A, lower panel). Then, these samples were blotted by an anti-HA antibody. Although, the predicted molecular size of Edg-6/S1P4 from the cDNA sequence is 42 kDa, the Edg-6/S1P4-in-the-cell surface fractions appeared at a molecular weight of 55–65 kDa, suggesting post-translational modification (Fig. 2A, arrowhead). In light of previous reports showing that some seven-transmembrane-spanning receptors are glycosylated in their N-terminal portion (Ulloa-Aguirre *et al.* 1999), we

performed endoglycosidase digestions on the samples, using PNGase F (peptide N-glycosidase F). The molecule had an increased mobility after these treatments (Fig. 2A, *). That the molecule is a non-glycosylated form of Edg-6/S1P4, and not a degraded product, was unquestionable, because it could be detected by both anti-FLAG and anti-HA antibodies (data not shown). The Endo H (endoglycosidase H)-sensitive molecule (Fig. 2A, arrow) indicates the high-mannose type oligosaccharide-conjugated form of Edg-6/S1P4, which exists in the ER. These results suggest that the Edg-6/S1P4 which is localized at the cell surface has undergone

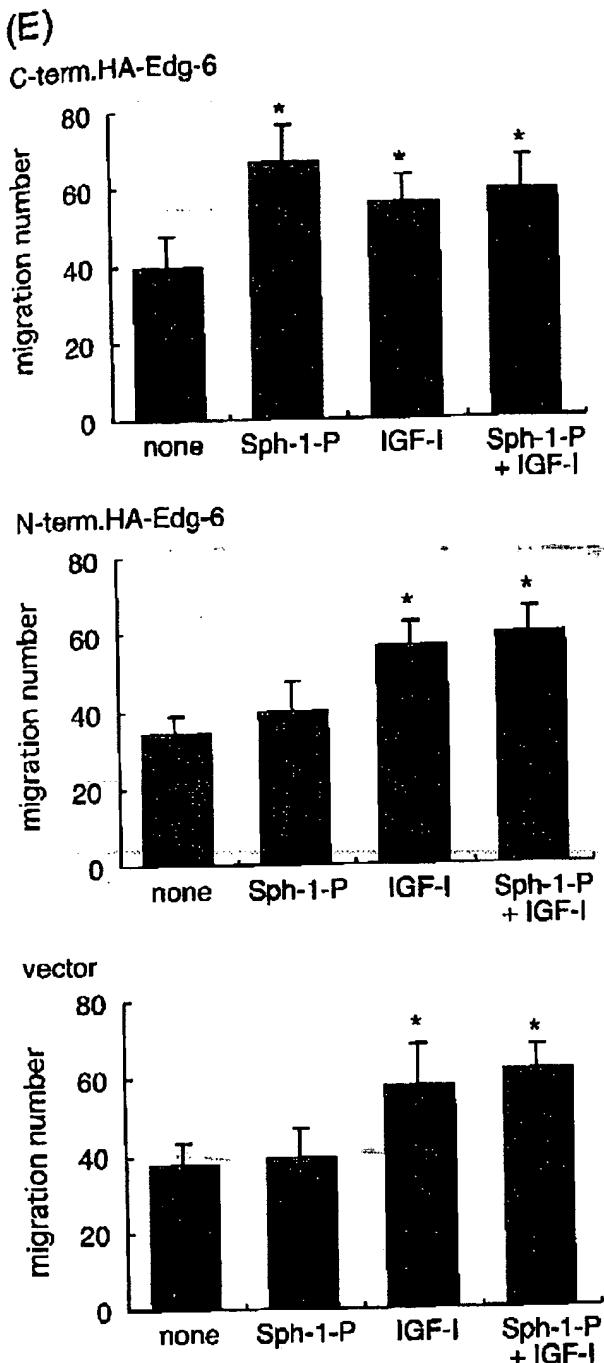


Figure 1. Continued.

PNGase F-sensitive oligosaccharide modification at the Golgi.

To further analyse the cell surface localization of Edg-6, we measured the specific binding of radiolabelled ligand in Edg-6/S1P4-expressing CHO cells. Figure 2B

shows that Edg-6/S1P4-expressing cells bind [³²P]-Sph-1-P specifically. Scatchard analysis for Edg-6/S1P4-expressing cells indicated a dissociation constant (K_d) of 16.7 nM and a maximum binding capacity (B_{max}) of 620 fmol per 1×10^5 cells.

Next, we examined whether Sph-1-P might regulate cell migration in the Edg-6/S1P4 stable transfectants. As shown in Fig. 3A, increased Sph-1-P-induced cell migration was observed in Edg-6/S1P4-expressing cells compared to control cells. Further enhancement of cell migration through Edg-6/S1P4 was not obtained in the presence of both Sph-1-P and IGF-I in the lower chamber. To confirm that the Sph-1-P-mediated cell migration was not influenced by a clonal effect in the stable transfectants, we examined two clones (left and right panels). Then, we examined whether Sph-1-P treatment led to enhanced cell migration via Edg-6/S1P4 in these cells in a dose-response manner. As shown in Fig. 3B, significant Sph-1-P-induced cell migration was observed in Edg-6/S1P4-expressing cells, compared to control cells. Because further enhancement of the cell migration was observed in a high dose range, typical bell-shaped curves were not obtained. The presence of Sph-1-P likely does not affect the cell-to-extracellular matrix adhesive properties (data not shown). Taken together, these results demonstrate that the presence of Sph-1-P significantly led to enhanced cell migration via Edg-6/S1P4 in CHO stable transfectants.

Pertussis toxin inhibits Sph-1-P-induced cell migration mediated through Edg-6/S1P4

Many chemokines induce cell migration through specific seven-transmembrane-spanning receptors (Baggiolini 1998). Such migration is completely abolished by the Gi-specific inhibitor pertussis toxin (PTx). Additionally, the phosphatidylinositide 3-kinase (PI3K) inhibitor LY294002 and the mitogen-activated protein kinase (MAPK) inhibitor PD98059 also have the same effect (Curnock *et al.* 2002). Therefore, we examined whether these inhibitors influenced the Sph-1-P-induced cell migration that is mediated through Edg-6/S1P4. PTx (500 ng/mL) completely abolished Sph-1-P-induced cell migration, whereas IGF-I-mediated cell migration was not affected (Fig. 4). Significant inhibition was not observed with the other inhibitors, although the trypsinized cells were only pre-incubated for 10 min with the inhibitors in these experiments (Experimental procedures), so we might not have been able to obtain a sufficient effect in such a short time. These results suggest that the Gi protein-coupled signalling machinery is involved at least in the signal transduction pathways

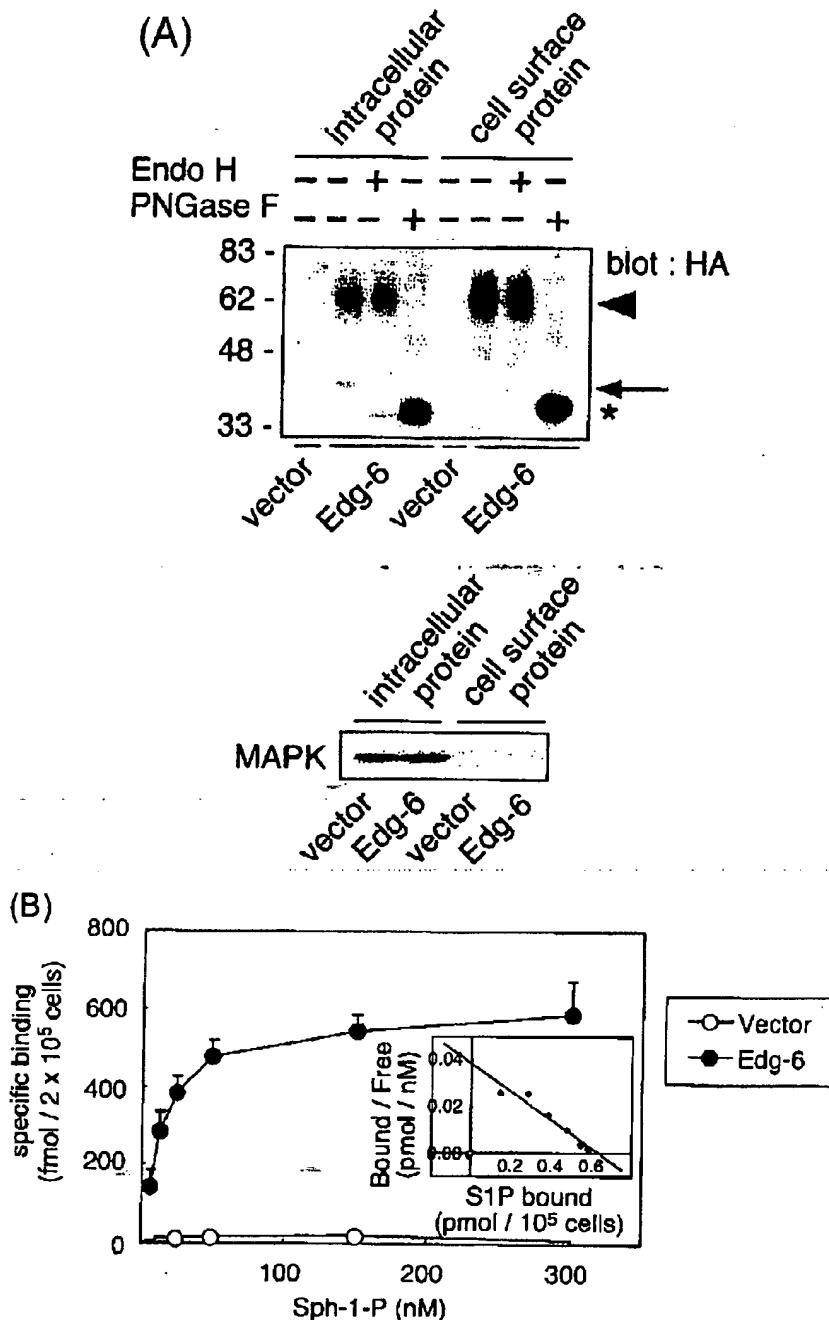


Figure 2 Establishment of cells stably expressing Edg-6 at the surface. (A) Cell surface proteins of CHO cells (4×10^5), stably transfected with vector or Edg-6/S1P4 tagged with an N-terminal FLAG and C-terminal HA, were biotinylated with sulfo-NHS-SS-hiotin and separated from the cell lysates by streptavidin-agarose beads. These samples were treated with endoglycosidase, Endo H or PNGase F. All samples were separated on SDS-PAGE, followed by Western blotting using an anti-HA antibody (upper panel). The arrowhead indicates the cell surface-associated Edg-6/S1P4, and the arrow indicates the Edg-6/S1P4 modified by a high mannose-type oligosaccharide. The asterisk indicates the full length Edg-6/S1P4 from which all N-linked oligosaccharides were truncated. In order to prove that intracellular proteins were not biotinylated, these intracellular and cell surface fractions were blotted using an anti-MAPK antibody (lower panel). (B) 2×10^5 of Edg-6/S1P4-expressing CHO cells (closed circle) or vector-expressing CHO cells (open circle) were grown on plastic dishes followed by incubation with ice-cold binding buffer. These cells were treated with the indicated concentrations of [32 P] Sph-1-P in binding buffer. After incubation for 30 min at 4°C , the cells were lysed with extraction buffer and bound [32 P] Sph-1-P was quantified by scintillation counting, as described under Experimental procedures. Specific binding of [32 P] Sph-1-P was calculated as the binding of Edg-6-expressing cells minus the binding of vector-expressing cells. These data represent the average of four independent experiments, with error bars indicating the SD. The inset shows the Scatchard plot of [32 P] Sph-1-P binding to the cells.

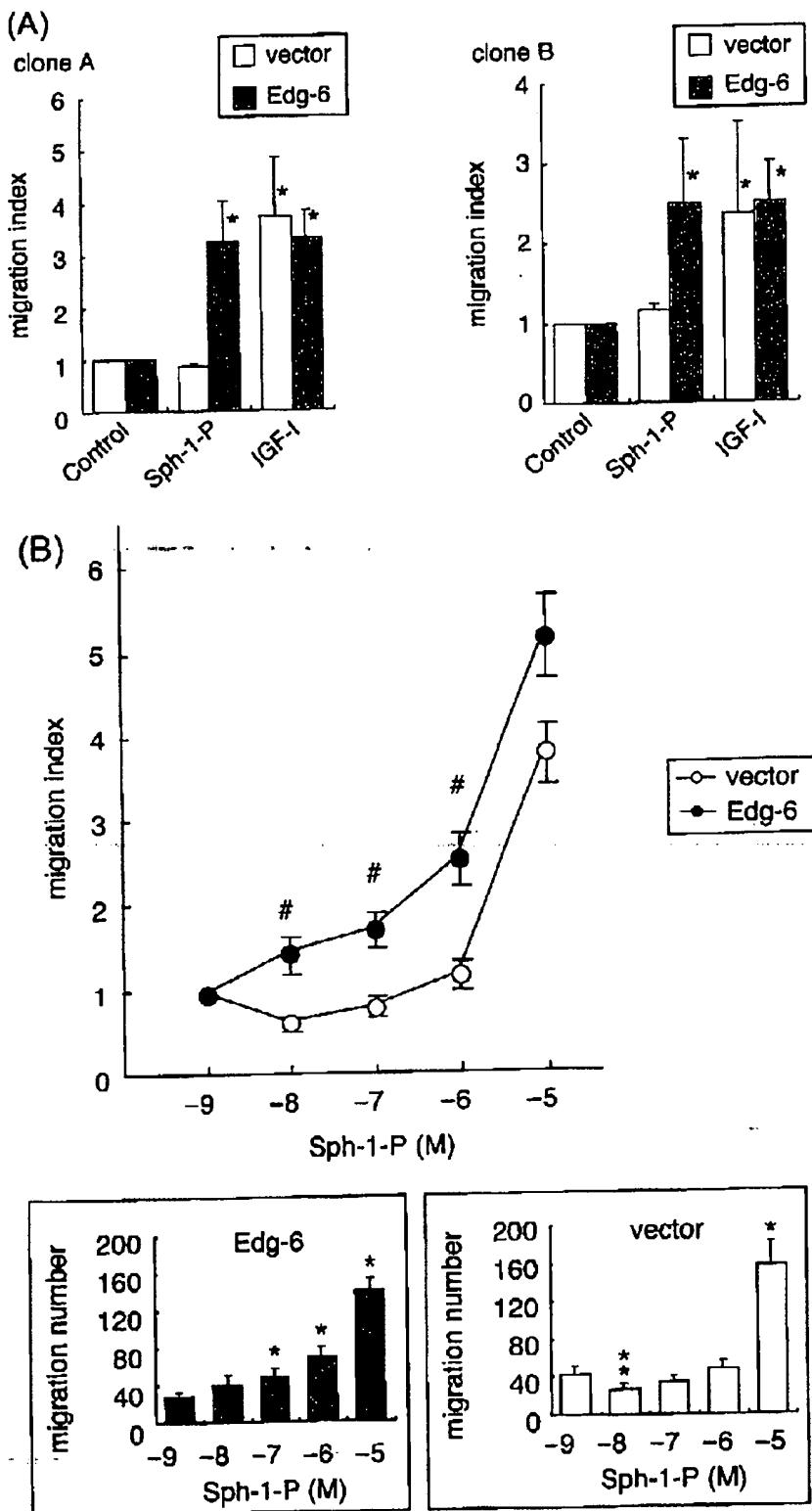
of Sph-1-P-induced cell migration that is mediated through Edg-6/S1P4 in CHO cells.

Sph-1-P stimulation via Edg-6/S1P4 induces transient activation of Cdc42 but not Rac

A recent report indicated that the regulation of cell motility involves activation of the Rho family of small GTPases (Hall 1998). It has also been reported that

Sph-1-P-mediated cell migration involves the activation of Rac in Edg-1/S1P1- or Edg-3/S1P3-expressing cells, and the inhibition of Rac in Edg-5/S1P2-expressing cells (Okamoto *et al.* 2000). We, therefore, examined whether these GTPases are involved in an enhancement of Edg-6/S1P4-mediated cell migration. To analyse the activation of Cdc42 or Rac in Edg-6/S1P4- or vector-expressing CHO cells following Sph-1-P stimulation, we carried out a pull-down assay using GST-PBD. The

Figure 3 Sph-1-P stimulation leads to enhanced cell migration via Edg-6/S1P4 expressed on the cell surface in CHO stable transfectants. (A) CHO cells (2×10^5) stably expressing Edg-6/S1P4 (closed) or vector (open) were trypsinized and then added to the upper well of the Transwell chamber, and 1 μ M Sph-1-P or 10 ng/mL IGF-I was placed into the lower chamber. The migration assay was performed as described under Experimental procedures. The migration index for the experimental wells was calculated as the number of transmigrated cells in chambers with chemoattractants divided by the number of transmigrated cells in control wells containing no chemoattractants. There were no significant differences between the two transfectants in the numbers of cells migrated in control wells. These data represent the average of three independent experiments with error bars indicating the SD. The same data were obtained in assays using another clone (left and right panels). Statistical significance: * $P < 0.01$ vs. control. (B) Sph-1-P treatment led to enhanced cell migration via Edg-6/S1P4 in a dose-response manner. CHO cells (2×10^5) expressing Edg-6/S1P4 (closed) or vector (open) were added to the upper well of a Transwell chamber, and the indicated concentrations of Sph-1-P were placed into the lower chamber. The migration assay was performed as discussed in (A). These data represent the average of three independent experiments with error bars indicating the SD. Statistical significance: # $P < 0.001$ vs. vector. The lower graphs represent the number of transmigrated cells. These data represent the average of three independent experiments with error bars indicating the SD. Statistical significance: * $P < 0.001$, ** $P < 0.05$ vs. 10 $^{-9}$ M Sph-1-P.



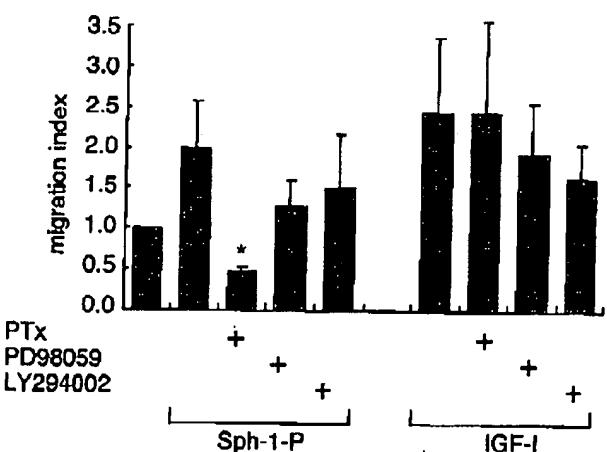


Figure 4 Pertussis toxin inhibits Sph-1-P-induced, Edg-6/S1P4-mediated cell migration. Edg-6/S1P4-expressing CHO cells were suspended by trypsinization, and preincubated with 500 ng/ml PTx, 50 μ M LY294002, or 50 μ M PD98059 at 37°C. Then, 1 μ M Sph-1-P- or 10 ng/ml IGF-I-induced cell migration was determined as described under Experimental procedures. These data represent the average of three independent experiments with error bars indicating the SD. Statistical significance: * $P < 0.01$ vs. Sph-1-P.

results in Fig. 5A,B indicate that Cdc42 was transiently activated upon Sph-1-P stimulation in the Edg-6/S1P4-expressing cells, but not the vector-expressing cells. This activation behaved in a PTx-sensitive manner (data not shown). On the other hand, Rac activation was not observed in these conditions (Fig. 5C,D). These results led us to conclude that the transient activation of Cdc42, through Gi-mediated signal transduction pathways, might be implicated in cell migration induced by Sph-1-P and mediated through Edg-6/S1P4.

Cdc42 activation is required for Sph-1-P-mediated cell migration

We examined the migration in cells transfected with the dominant negative or constitutively active form of Cdc42. CHO cells stably expressing Edg-6/S1P4 were transiently transfected with myc-Cdc42, myc-N17 Cdc42 (the dominant negative form), myc-V12 Cdc42 (the constitutively active form), or the myc-vector. A migration assay was then performed. The results shown in Fig. 6 demonstrated that few cells expressing N17 Cdc42 migrated upon Sph-1-P stimulation, whereas IGF-I-induced cell migration was observed (Fig. 6D). However, no apparent difference was observed among vector-, wild-type-, and V12 Cdc42-expressing cells upon Sph-1-P stimulation (Fig. 6A,B,C). Furthermore,

there was no apparent difference in the Sph-1-P-mediated cell migration between the dominant negative Rho (N19 Rho) and Rac (N17 Rac)-expressing Edg-6/S1P4 stable transfectants (data not shown). Therefore, the enhancement of cell migration observed upon stimulation by Sph-1-P and mediated through Edg-6/S1P4, required the activation of Cdc42.

Discussion

The seven transmembrane-spanning receptors are able to bind their ligand only when these receptors are expressed at the cell surface. The γ -aminobutyric acid receptor type B, GABA_B, is known to have difficulty localizing to the cell surface and is retained on intracellular membranes when expressed in cultured cells (Couve et al. 1998). It has been clearly shown that GABABR1 forms heterodimers with GABABR2, and this dimerization is required for expression on the cell surface (Jones et al. 1998; Kaupmann et al. 1998; White et al. 1998). In this study, we found that Edg-6/S1P4-GFP and N-terminal-tagged Edg-6/S1P4 had difficulty localizing to the cell surface (Fig. 1). Interestingly, once a FLAG tag was introduced at the N-terminus of the C-terminal GFP-tagged Edg-6/S1P4, the fusion protein localized to the cell surface (Fig. 1C). On the other hand, the N-terminal FLAG-tagged Edg-6 was not expressed at the cell surface, as described in the results. Moreover, we demonstrated that a PNGase F-sensitive oligosaccharide was fused to Edg-6/S1P4, larger than that found on another Sph-1-P receptor Edg-1/S1P1 (Kohno et al. 2002). For a seven transmembrane-spanning receptor, export from the ER to the Golgi represents the limiting step in cell surface expression (Petaja-Repo et al. 2000). With this in mind, we considered that the position and molecular size of tags fused with Edg-6/S1P4 could significantly affect its glycosylation at the Golgi, but we scarcely understood its precise mechanisms. Because commercially available antibodies did not react in immunoprecipitation, immunoblotting, or immunofluorescence microscope assays of Edg-6/S1P4 under our experimental conditions (data not shown), it is still uncertain whether non-tagged or endogenously expressed Edg-6/S1P4 is localized at the cell surface.

It is known that Sph-1-P is released from activated platelets (Yatomi et al. 1995). However, much Sph-1-P is present in blood (Murata et al. 2000). With this in mind, we reasoned that there might be some mechanism for regulating the receptor expression or signal transduction of Sph-1-P receptors expressed on the surface of the lymphocytes. In vascular endothelial cells, which express Edg-1/S1P1 and Edg-3/S1P3, the expression of these

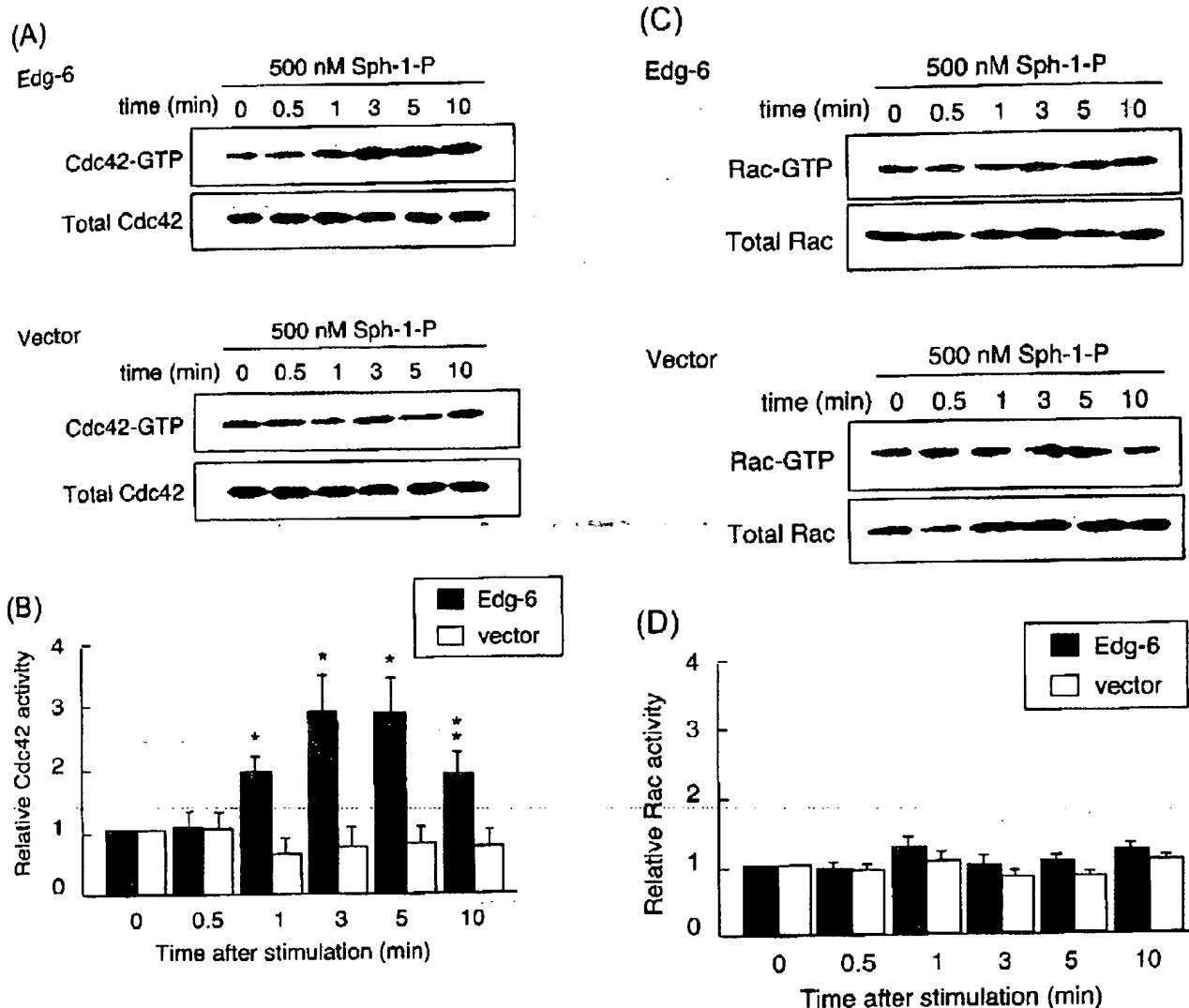


Figure 5 Sph-1-P stimulation via Edg-6/S1P4 induces transient activation of Cdc42 but not Rac. A time course of Cdc42 and Rac activation mediated through Edg-6/S1P4 was examined in CHO cells. CHO cells (4×10^6) stably expressing Edg-6/S1P4 or vector were grown on culture dishes, followed by serum starvation for 36 h, and then stimulated with 500 nM Sph-1-P. At the indicated times, cell lysates were prepared, and these were used for an affinity precipitation assay for 45 min at 4 °C in the presence of GST-PBD and glutathione Sepharose beads. GTP-Cdc42 or GTP-Rac bound to the beads was collected, and solubilized with Laemmli sample buffer. The proteins were separated on SDS-PAGE and analysed by Western blotting using an anti-Cdc42 (A) or an anti-Rac (C) antibody. These results were quantified by densitometry using the NIH image program, and the relative activity was calculated, using as a standard the activity in the absence of Sph-1-P (B, D). The blots illustrated represent a typical blot out of four, and the graphs represent the average of four independent experiments with error bars indicating the SD. Statistical significance: * $P < 0.01$; ** $P < 0.05$ vs. vector.

receptors reportedly is induced upon TPA stimulation (Hla & Maciag 1990). However, the mechanisms regulating this expression are unclear. Recent reports indicate that the expression of Edg-6/S1P4 in CD4-T cells is decreased during differentiation, as is that of many chemokine receptors (Graefel & Goetzl 2002). In this study, we suggest that the localization of Edg-6/S1P4 to

the cell surface might be regulated by some mechanism(s) (Figs 1 and 3). Therefore, it will certainly be important in the future to elucidate mechanisms that regulate the cellular localization of Edg-6/S1P4.

Of the Edg-family Sph-1-P receptors, CHO cells express mRNA for only Edg-5/S1P2 (Okamoto *et al.* 1998). It has been reported that Sph-1-P exhibits inhibitory

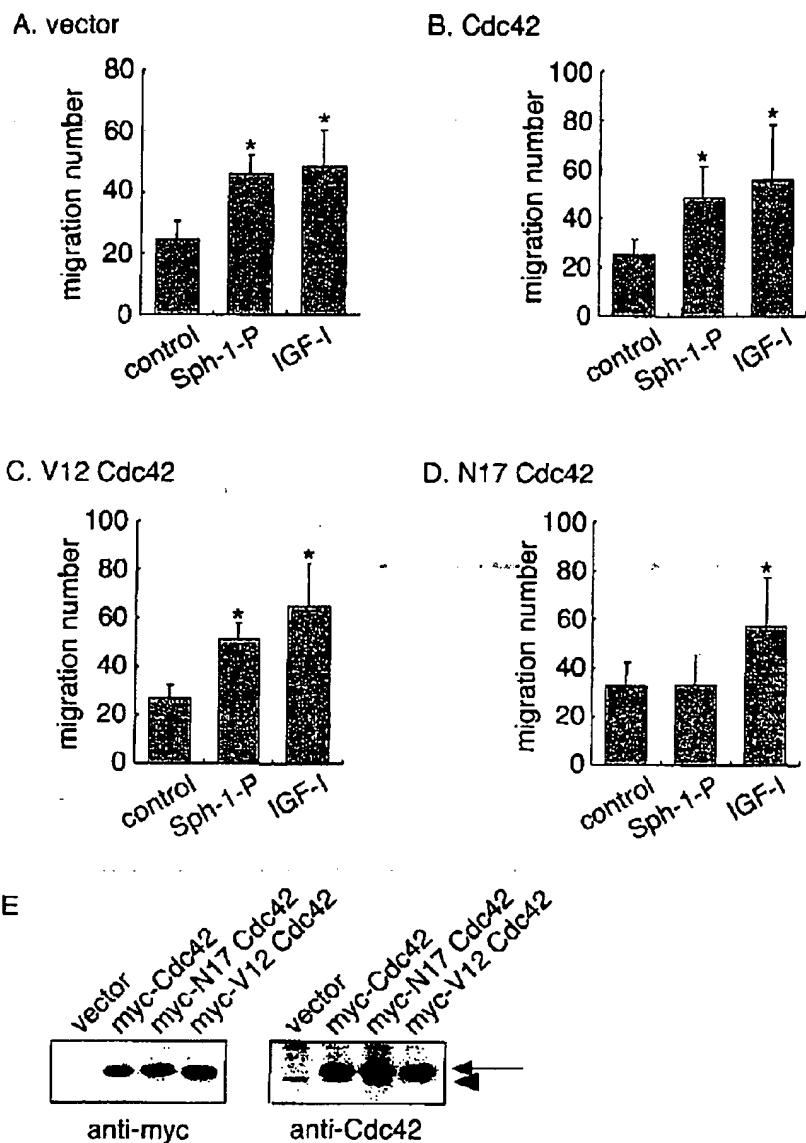


Figure 6 Cdc42 activation is required for Sph-1-P-mediated cell migration. Edg-6/S1P4-expressing CHO cells were transiently transfected with pcDNA3 alone (A), myc-Cdc42 (B), myc-V12 Cdc42 (constitutive active, C) or myc-N17 Cdc42 (dominant negative, D). After transfection for 24 h, the cells (2×10^5) were added to the upper well of the Transwell chamber, and 1 μ M Sph-1-P or 10 ng/ml IGF-1 was added into the lower well, followed by incubation for 4 h at 37 °C. These data, analysed as in Fig. 3, represent the average of three independent experiments with error bars indicating the SD. Statistic significance: * $P < 0.01$ vs. control. (E) The expression of Cdc42 in these transfected cells was analysed by Western blot using an anti-myc (left) or an anti-Cdc42 (right) antibody. The arrow indicates the myc-tagged Cdc42. The arrowhead indicates endogenous Cdc42. These results demonstrate that Sph-1-P-induced, Edg-6/S1P4-mediated cell migration requires the activation of Cdc42.

regulation for cell migration in Edg-5/S1P2-overexpressing CHO cells (Okamoto *et al.* 2000). In contrast, another report provides evidence that the activation of the Rho family small GTPase, Rac, is involved in cytoskeletal rearrangement and cell migration in Sph-1-P-stimulated, Edg-1/S1P1- or Edg-3/S1P3-expressing cells (Lee *et al.* 1999). In our study Sph-1-P-mediated cell migration was observed in Edg-6/S1P4-overexpressing CHO cells (Figs 1 and 3), and specific Cdc42 activation occurred in these cells upon stimulation with Sph-1-P (Fig. 5). Furthermore, an enhancement in migration of Edg-6/S1P4-expressing cells in the presence of Sph-1-P was completely abolished by the coexpression of a dominant negative Cdc42 (Fig. 6). Cell

migration involving Cdc42 activation is consistent with a previous report (Allan *et al.* 1998). However, as shown in Fig. 6, further activation was not observed when a constitutively active form of Cdc42 was transfected in these cells. Therefore, other factors may be involved in cell migration induced through Edg-6/S1P4 by Sph-1-P stimulation.

In this study, we found that the regulation of cell migration through Edg-6/S1P4 involves pertussis toxin-sensitive, Gi protein-coupled, signalling cascades (Fig. 4). Very recently, it was reported that the Gα15 (Gna15) protein is located in tandem just upstream of Edg-6/S1P4 on the same chromosome, and that these proteins may be coexpressed in the same tissues, such as

spleen and lung, as indicated by Northern blot analysis (Contos *et al.* 2002). These findings strongly suggest that Edg-6/S1P4 may interact with Gα15 at physiological conditions in lymphocytes. Therefore, Edg-6/S1P4 may perform a different role in these cells than in Edg-6/S1P4-expressing CHO cells which also express Gαi. Further studies will be necessary to elucidate a physiological role for Edg-6/S1P4, especially to clarify the functions of Edg-6/S1P4 signalling pathways when the receptor forms a complex with the Gα15 protein.

Experimental procedures

Reagents and antibodies

ν -erythro-sphingosine-1-phosphate (Sph-1-P) was purchased from Matreya (Pleasant Gap, PA, USA). A commercial protease inhibitor cocktail (complete, EDTA free) was from Roche (Roche, Mannheim, Germany), and recombinant human insulin-like growth factor-I (IGF-I) from Peprotech EC (London, UK). Pertussis toxin was from Sigma (St Louis, MO, USA). Endo H (endoglycosidase Hf), Peptide N-glycosidase F (PNGase F) and anti-MAPK antibody were all purchased from Cell Signalling (Beverly, MA, USA). Anti-J11A antibody (Y-11) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-myc tag antibody from MBL (Nagoya, Japan), and anti-Cdc42 and anti-Rac antibodies from Transduction Laboratories (Lake Placid, NY, USA). Alexa 488 goat anti-rabbit IgG (H+L) conjugate, Alexa 594 phalloidin, Alexa 350 phalloidin, and ER-Tracker Blue-White DPX were from Molecular Probes (Eugene, OR, USA). Protein A-Sepharose fast flow, glutathione Sepharose 4B, anti-rabbit and anti-mouse IgG-(ab')₂ fragment, conjugated to horseradish peroxidase, and enhanced chemiluminescence (ECL-plus) detection kits were from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

DNA constructs

Full length mouse Edg-6/S1P4 coding sequences were amplified from an ES⁺ clone, AI158066, which lacks two base pairs at the initiation codon, using Ex Taq polymerase (Takara, Shiga, Japan) with primers (sense, 5'-GAAGATCTATGAACATCAGTAC CTGGTCC-3'; anti-sense, 5'-AGCCTCCGCAGCACCA GATCITC-3') containing the initiation codon. The cycling parameters were an initial denaturation step of 1 min at 96 °C, followed by 25 cycles of denaturation at 96 °C for 15 s, annealing at 50 °C for 2 s, and extension at 74 °C for 30 s. The polymerase chain reaction introduced a BglII site at both the 3' and 5' ends of the Edg-6/S1P4-cDNA. PCR products were gel purified, then ligated into pGEM-T (Promega, Madison, WI, USA), and sequenced on both strands. The Edg-6/S1P4 fragment was excised by BglII from pGEMT-Edg-6/S1P4, then directly cloned into the BamHI site in the multiple cloning site of the pEGFP N1 vector (Clontech, Palo Alto, CA, USA) or pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). Other cDNAs such as Edg-1/S1P1, Cdc42, N17 Cdc42, V12 Cdc42 and GST-PBD were obtained by

similar methods to those described above, and were provided by A. Wada and K. Ohta (this laboratory).

Cell culture and transfections

Jurkat cells were cultured in RPMI medium (Sigma), supplemented with 10% foetal bovine serum (FBS) (Iwaki, Chiba, Japan). NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) with 10% FBS. Chinese hamster ovary (CHO) cells were cultured in Ham's F-12 medium (Sigma) with 10% FBS. These cells were cultured at 37 °C in a humidified 5% CO₂ atmosphere. The cells were transfected using Effectene (Qiagen, Hilden, Germany), LipofectAMINE (Invitrogen), or LipofectAMINE Plus kits according to their manufacturers' instructions. Stably transfected clones expressing epitope-tagged Edg-6/S1P4 were selected in medium containing 600 µg/mL Geneticin (Sigma).

Sph-1-P binding assay

[³²P] Sph-1-P was synthesized enzymatically using recombinant sphingosine kinase (SK). The cDNA of the murine SK1a was cloned into the bacterial expression vector pMAL-c2X and was expressed in *Escherichia coli* as a fusion protein with maltose binding protein (MBP). The purified fusion proteins were isolated from amylose resin with 10 mM maltose. [³²P] Sph-1-P was synthesized using [γ -³²P] ATP, sphingosine and MBP-SK fusion protein. The cells (2×10^6) were washed twice with ice-cold binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 15 mM NaF and 0.1% (w/v) fatty acid free BSA (Sigma)) and incubated with the indicated concentrations of [³²P] Sph-1-P in binding buffer. After incubation for 30 min at 4 °C, the cells were lysed with extraction buffer (0.1% SDS, 0.4% NaOH and 2% Na₂CO₃) and bound [³²P] Sph-1-P was quantified by scintillation counting.

Immunofluorescence microscopy

Cells were grown on glass coverslips (Matsunami, Kyoto, Japan). After transfection for 24 h, the cells were washed twice with phosphate buffered saline (PBS) and fixed with 3.7% formaldehyde in PBS at room temperature for 20 min. Permeabilization was performed in 0.5% saponin in 1 mg/mL BSA/PBS for 5 min. Immunostaining was performed by incubation with a 1 : 200 dilution of the anti-HA antibody, followed by detection with a 1 : 400 dilution of Alexa 488 anti-rabbit IgG conjugate at room temperature for 1 h. The cells were washed three times with PBS after each incubation. The coverslips were rinsed in water and mounted on to glass slides using Mowiol 4-88 (Calbiochem, San Diego, CA, USA). The cell images were digitally captured from a Zeiss AxioSkop 2 plus (Carl Zeiss, Thornwood, NJ, USA) microscope using an Axiocam CCD camera (Carl Zeiss).

Cell surface biotinylation and endoglycosidase digestions

CHO cells (4×10^6) stably expressing Edg-6/S1P4, tagged with both N-terminal FLAG and C-terminal HA, were grown on

culture dishes. The cells were washed twice with cold PBS, and the cell surface proteins were biotinylated by incubation with 1 mg/mL sulfo-NHS-SS-biotin (Pierce, Rockford, IL, USA) at 4 °C for 1 h. The excess reagent was washed three times with a quenching buffer (50 mM Tris-HCl, 150 mM NaCl) at 4 °C for 5 min. Cells were lysed with extraction buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 5 mM sodium orthovanadate, 1% NP-40 and protease inhibitors) for 5 min at 4 °C, and sonicated (10 × 6 s). The cell lysates were centrifuged at 16 000 g at 4 °C for 10 min. Then, the supernatants were removed and incubated with streptavidin-agarose beads (Sigma) at 4 °C for 2 h to separate the cell surface and intracellular proteins. The precipitates were then collected by centrifugation at 8000 g at 4 °C for 40 s, and washed three times with Lubrol buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.5 mM EDTA, 0.1% Lubrol (PX)). The cell surface proteins and the intracellular fractions remaining in the supernatants were resuspended in SDS-PAGE sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol) and boiled for 5 min. The samples were then renatured with 1% NP-40-containing buffer (50 mM sodium phosphate, pH 7.5), and endoglycosidase digestions were performed at 37 °C for 2 h, according to the manufacturer's recommended procedure. The reactions were stopped by adding 4× SDS-PAGE sample buffer. These samples were analysed by Western blot as described.

Cell migration assay

Chemotactic cell migration was assessed using modified Boyden chambers with Transwell filters (6.5 mm diameter, 8 µm pores; Corning Costar Corp., Cambridge, MA, USA). Before beginning the assay, both the upper and lower filter surfaces were coated with 100 µg/mL porcine collagen type I-C (Nitta gelatin, Osaka, Japan) for 1 h at room temperature. The filters were then washed with Ham's F-12 medium and air-dried. The bottom chambers were filled with Ham's F-12 medium containing 0.1% fatty acid-free bovine serum albumin (BSA). CHO cells were harvested by treatment with 0.25% trypsin-EDTA (Sigma) and then resuspended in Ham's F-12 with 0.1% fatty acid-free BSA. Cells (2×10^5) were added to the upper chambers of the Transwell filters, and medium containing the indicated chemoattractants was added to the lower chamber, then the cultures were incubated at 37 °C in a humidified CO₂ incubator. After 4 h, cells remaining on the upper surface of the filter were removed with a cotton swab. Cells on the lower side of the Transwell filter were fixed with cold methanol and stained with 1% crystal violet in 2% ethanol. In experiments where inhibitors were used, the trypsinized cells were suspended and preincubated with the indicated inhibitors for 10 min prior to loading the chambers, and the inhibitors were placed into both the upper and lower chambers during the incubation. The number of cells migrating to the lower surface of the Transwell filter was determined by counting the number of cells in five random fields. The migration index was calculated as the number of transmigrated cells in experimental wells divided by the number of cells transmigrating toward chemoattractant-free medium in control wells.

Electrophoresis and immunoblotting

Reducing SDS-PAGE was performed on 10% or 12% polyacrylamide gels. After electrophoresis, the proteins were transferred to a PVDF membrane (Millipore, Bedford, MA, USA). Blocking was performed with 5% skim milk in TBS-T (20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.05% Tween-20) or 0.5% BSA in TBS-T for 1 h at room temperature. This was followed by incubation overnight at 4 °C with the primary antibody. The blots were then washed with TBS-T and incubated with the secondary antibody for 1 h at room temperature. The blots were washed again with TBS-T, developed with ECL-plus, and quantified by densitometry with the NIH Image program. When necessary, the antibodies were stripped off the membranes by incubation in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS) for 30 min at 50 °C with constant agitation, rinsed twice in TBS-T, and then reprobed with other antibodies as indicated.

Pull-down assay

A pull-down assay was performed as previously described (Benard et al. 1999). CHO cells (4×10^6) were grown on the culture dishes, followed by serum starvation for 36 h, and then stimulated with 500 nM Sph-1-P. After the indicated times, these cells were gently washed with cold PBS, and proteins were extracted with lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM MgCl₂, 1% NP-40, 10% glycerol, 1 mM PMSF, and protease inhibitors). The lysates were incubated with a purified glutathione S-transferase (GST)-PAK1-binding domain (amino acids 67–150) fusion protein and glutathione Sepharose 4B at 4 °C for 15 min. The precipitates were collected by centrifugation at 8000 g at 4 °C for 40 s, washed four times with lysis buffer, then solubilized in SDS-PAGE sample buffer. These samples were then analysed by Western blot using an anti-Cdc42 or an anti-Rac antibody, and quantified by densitometry with the NIH Image program.

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